# REDUCTION OF CARBOXYLIC ACIDS TO ALCOHOLS USING SYNGAS AND *CLOSTRIDIUM LJUNGDAHLII* AS BIOCATALYST

A Thesis

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by

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# ABSTRACT

Short-chain carboxylic acids generated by various mixed- or pure-culture fermentation processes have been considered valuable precursors for production of bioalcohols. While conversion of carboxylic acids into alcohols is routinely performed *via* catalytic hydrogenation or with strong chemical reducing agents, biological conversion routes are being explored. Reduction of *n*-butyric acid by pure cultures using glucose as the source of energy and electrons have been studied. However, the high cost of sugars has lead to the searching for cheaper sources. Syngas produced *via* biomass gasification, which is a blend of carbon monoxide, hydrogen and carbon dioxide, was studied as an economical source of energy and electrons with pure cultures of *Clostridium ljungdahlii* as a biocatalyst for the carboxylic acids reduction. Acetic acid, propionic acid, *n*-butyric acid, isobutyric acid, *n*-valeric acid, and *n*-caproic acid were successfully converted into their corresponding alcohols. Furthermore, biomass yields and fermentation stoichiometry from our experimental data enabled us to amend thermodynamic calculations with the goal to evaluate how much metabolic energy *C. ljungdahlii* can generate during fermentation of carbon monoxide. Our results show ATP yield of 0.42 ATP per carbon monoxide molecule consumed for fermentation of syngas, and 0.38 ATP per carbon monoxide molecule consumed when *n*-butyrate is added to the growth medium. The ratio of protons pumped across the cell membrane *vs*. electrons transferred from ferredoxin to $NAD^+$ *via* the RNF complex is suggested to be 1.0. The results obtained in this work suggest that the production of bioalcohols, based on the reduction of carboxylic acids, may be an attractive alternative industrial process.

# BIOGRAPHICAL SKETCH

Jose Miguel Pérez was a MSc candidate in Biological and environmental Engineering. He obtained the degree of Agricultural Engineering at Pontificia Universidad Catolica de Chile in Santiago, Chile. After he obtained his professional degree he spent several years working in the fruit production sector in Chile.

# DEDICATION

To my father and my mother, as a present, in gratitude for their efforts in my education.

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# CHAPTER 1

# HYPOTHESIS

## *Introduction*

Increasing oil demand and limited discoveries of new reserves have stimulated the search of new liquid fuel sources and the development of new technologies. Since the development of transportation systems based on forms of energy other than liquid fuels has been very limited, liquid fuels still remain crucial for the trade and economy development ((IEA) 2011).

Efforts have been made to produce alternative liquid fuels mainly based on organic feedstocks which compete with food for arable land in which they are produced. Brazil has based its ethanol industry on the fermentation of sugars from sugar cane crops and USA is producing bioethanol from fermentation of corn starch (Engelhaupt 2008; Searchinger, Heimlich et al. 2008). However, those first generation bio-fuels are being questioned because of its interference with food markets. Therefore, the tendency is now to go towards second generation feedstocks, such as nonedible organic materials. One alternative is the fermentation of lignocellulosic biomass, which needs expensive pretreatments in to break down complex molecules, such as cellulose and hemicellulose, to simple fermentable sugars. However, large lignin fractions still remain after the process because of its low degradability.

Another promising technology that relies on nonedible feedstocks and that can convert even the most recalcitrant fraction of the biomass is the production of liquid fuels from syngas. This technology consists of gasification of biomass and further conversion into liquid fuels by

chemical or biological catalysis (van Steen and Claeys 2008; Balat 2009; Abubackar, Veiga et al. 2011). Biological catalysis has the advantage over chemical conversion that the former requires lower temperature and pressure in the process, biocatalists are more tolerant to sulfur species, and they have a higher specificity (Levy, Barnard et al. 1981; Grethlein and Jain 1992). Some microorganisms have been isolated that can grow autotrophically on carbon monoxide and produce acetic acid, *n*-butyric acid, ethanol, and *n*-butanol as fermentation products (Munasinghe and Khanal 2010). These organisms use the Wood-Ljungdahl pathway to synthesize acetyl-CoA, from which they build all biomass (Henstra, Sipma et al. 2007).

The most important biofuel produced is ethanol. However, ethanol chemical properties present some disadvantages over gasoline, such as lower energy density, which results in a lower gas mileage in vehicles, and its hygroscopicity and corrosivity. Other alcohols, such as *n*-butanol, with chemical properties closer to gasoline can be produced *via* fermentation. *n*-Butanol has been produced at commercial scale during the first half of the 20$^{th}$ century *via* fermentation of sugars by pure cultures of *Clostridium acetobutylicum* in a process called ABE fermentation (Jones and Woods 1986), however, it is not commercially viable anymore.

Improvement of ABE fermentation to favor the production of *n*-butanol has been studied by Richter et al. (2012) in which *n*-butyric acid is added to the fermentation medium and is reduced to the alcoholic form. Production of *n*-butyric acid and other carboxylic acids from lignocellulosic biomass has also been studied. Agler et al. (2011) proposed a fermentation system called the "carboxylate platform" in which production of short-chain carboxylic acids are favored by inhibition of methane-producing microorganisms. Significant concentrations of propionic acid, *n*-butyric acid, *n*-valeric acid, and *n*-caproic acid were obtained.

Reduction of carboxylic acids to the corresponding alcohols using glucose as the source of energy and electrons relies on feedstocks which compete with food crops for arable land. We propose a system in which the carboxylate platform provides the short chain carboxylic acids, which are then reduced to their corresponding alcohols using syngas fermenting organisms and syngas as the source of energy an electrons (Figure 1-1)

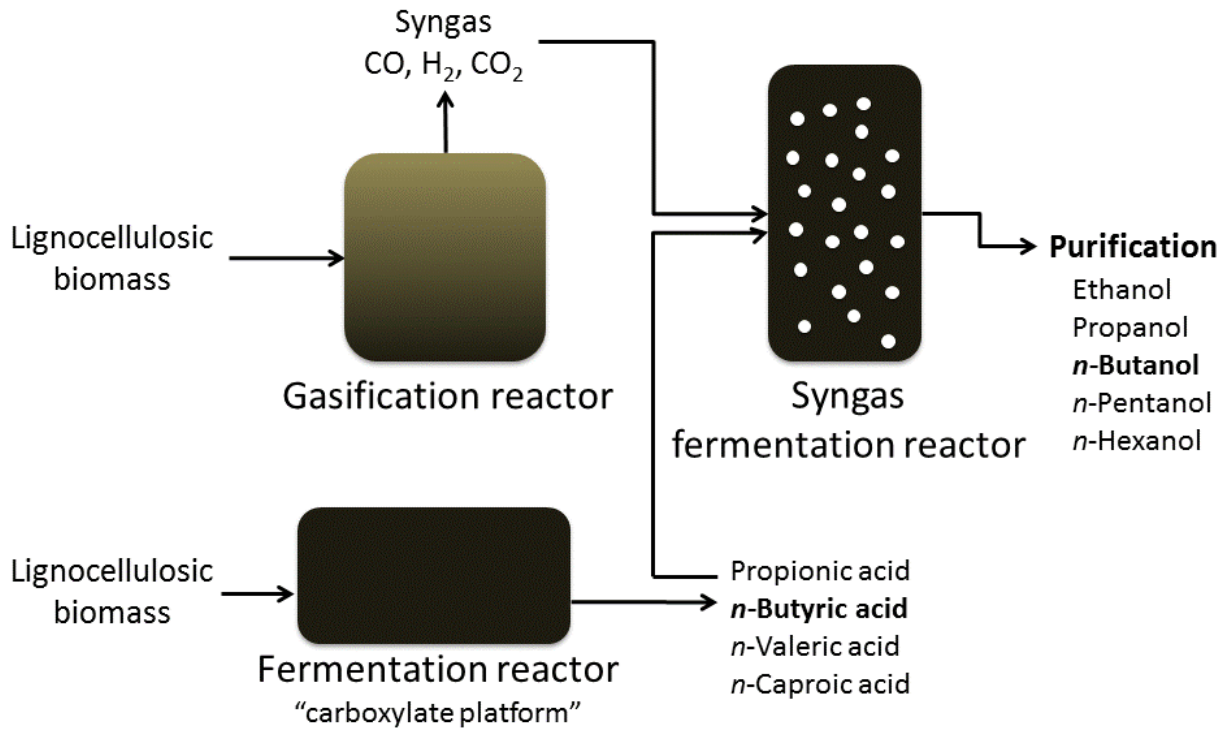



**Figure 1-1. Reduction of carboxylic acids to the corresponding alcohols using syngas fermentation**

*Clostridium ljungdahlii* is an acetogenic bacterium isolated from an enrichment inoculated with chicken waste (Tanner, Miller et al. 1993). It is able to grow autotrophically using carbon monoxide as energy and carbon source. It uses the Wood-Ljungdahl pathway to fix carbon in acetyl-CoA and its main products are acetic acid and ethanol. Some of the enzymes that catalyze the formation of intermediates for *n*-butyric acid and *n*-butanol production are not encoded in

*Clostridium ljungdahlii*'s genome; therefore, it cannot produce *n*-butanol from syngas. However, it contains the enzymes that can convert *n*-butanol into *n*-butyric acid (Köpke, Held et al. 2010) and those enzymes can work in the reverse pathway (Hüsemann and Papoutsakis 1989). No experimental data has been published about the specificity of those enzymes and the ability of *Clostridium ljungdahlii* to reduce organic acids of different molecular weight to the corresponding alcohols. The first aim of this study is to evaluate the ability of *Clostridium ljungdahlii* to reduce carboxylic acids of different molecular weight to the corresponding alcohols.

Energy for biosynthesis and product formation is obtained *via* the electron transfer from reduced ferredoxin to $NAD^+$ in the ferredoxin:$NAD^+$ oxidoreduction reaction (Fno) catalyzed by a membrane protein complex called Rnf complex, which uses the energy of the electrons transferred to translocate $H^+$ out of the membrane, generating a proton motive force. This proton motive force is then used by a $F_0F_1$ ATPase to synthesize ATP (Biegel and Muller 2010; Köpke, Held et al. 2010).

The difference in reducing potential between reduced ferredoxin and NADH is equivalent to -20 kJ/mol (Muller, Imkamp et al. 2008), and the energy necessary for pumping one mol $H^+$ out of the membrane was calculated to be from 8.90 to 9.87 kJ/mol (for an external pH between 4.50 and 6.50), based on membrane potential and delta pH calculated for *Clostridium acetobutylicum*, which is very close to *Clostridium ljungdahlii* (Huang, Gibbins et al. 1985). It means that theoretically two mol $H^+$ can be translocated out of the membrane with the energy of the Fno reaction. The second aim of this work is to study the stoichiometry of the syngas fermentation with and without the addition of a carboxylic acid (*n*-butyric acid in this case) to the growth medium, and the energy balance of syngas fermentation in *Clostridium ljungdahlii*.

***Hypotheses***

*Hypothesis 1:* Clostridium ljungdahlii *is able to convert short chain organic acids of different molecular weight into the corresponding alcohols using syngas as source of energy and reducing power*. Köpke et al. (2010) showed that *Clostridium ljungdahlii* can convert *n*-butanol into *n*-butyric acid when growing on fructose. Reactions involved in that pathway are reversible in other clostridia (Hüsemann and Papoutsakis 1989) and conversion of *n*-butyric acid to *n*-butanol is expected to be possible.

*Hypothesis 2: The Rnf complex in* Clostridium ljungdahlii *is able to pump 2* $H^+$ *out of the membrane per* $Fd_{red}$*:*$NAD^+$ *oxidoreduction reaction*. Theoretical calculations for ΔG of Fno reaction is -20 kJ/mol. ΔG for $H^+$ translocation out of the membrane in *Clostridium acetobutylicum* ranges between 8.90 and 9.87 kJ/mol (between pH 4.5 and 6.5). These data show that in that strain, which is relatively close to *Clostridium ljungdahlii*, it is thermodynamically possible to translocate 2 electrons per reaction.

# CHAPTER 2

# LITERATURE REVIEW

## *Syngas to liquid fuels by chemical catalysis*

Gasification of carbonaceous substrates results in a combination of gases that typically include CO, $H_2$, and $CO_2$ as the main components, and char and oil. The process is a thermal reaction and is performed at temperatures ranging from 200°C to 1000°C (Balat 2009) and a wide variety of substrates, such as wood, switchgrass, coal, and municipal waste, can be processed. The resulting composition of the gas depends on the substrate, the gasification technique, and the temperature of the process (Bridgwater 1995).

The first technology for conversion of syngas into liquid fuels was commercialized in Germany in the 1930s and became popular during World War II, when oil was needed and the abundant coal offered an opportunity for the production of liquid fuels. The technique, invented by Franz Fisher and Hans Tropsch, consists of a process using chemical catalysts and high pressure and temperature where the final product is a blend of different liquid hydrocarbons, such as methanol, acetic acid, methane, and heavy waxes (van Steen and Claeys 2008; Abubackar, Veiga et al. 2011). Fisher-Tropsch synthesis needs a specific $CO/H_2$ ratio and conditioning of the gas, to keep the required ratio, which is one of the most relevant operational costs. Also, the syngas needs purification of sulfur compounds to avoid catalyst poisoning. All processes from gasification to final purification require different conditions and take place in different units. It results in a complex and expensive process (Abubackar, Veiga et al. 2011).

### *Biological catalysis*

Microbial catalysis for syngas conversion into liquid fuels presents some advantages over chemical catalysis. First, the reaction is not affected by the CO:$H_2$ ratio, which is variable depending on the gasification process and the substrate used. Second, microbial catalysis is more resistant to catalyst poisoning by sulfur gases, and therefore the cost of gas cleanup before the conversion can be reduced. Third, since the biological process is performed at lower temperatures and pressures, less energy is required in the process. Fourth, biological catalysts are more specific and environmental conditions can be modified to favor a specific end product (Vega, Klasson et al. 1990; Worden, Grethlein et al. 1991; Bredwell, Srivastava et al. 1999).

The first researchers who reported the ability of homoacetogens to use CO as energy and carbon source were Dr. Rathin Datta and Dr. Greg Zeikus in 1981 (Lynd, Kerby et al. 1982). Since then, several mesophilic and thermophilic microorganisms, which can grow autotrophically on CO, have been isolated. The main products detected are acetate, butyrate, ethanol and butanol (Henstra, Sipma et al. 2007; Munasinghe and Khanal 2010). Most microorganisms identified produce acetate and *n*-butyrate as end products. Some of them can also produce ethanol and *n*-butanol (Table 2-1).

#### *Clostridium ljungdahlii*

*Clostridium ljungdahlii* was isolated from chicken yard waste in an enrichment at initial pH of 5, and incubated at 37°C. The atmosphere used was composed of 75% CO, 15% $H_2$, 10% $CO_2$, and 2% $CH_4$ (Barik, Prieto et al. 1988). The strain was deposited in the American Type Culture Collection with the name strain ATCC $49587^T$. It is a gram positive, rod shaped, spore forming,

and motile bacterium and can grow autotrophically in $CO_2$-$H_2$ or CO under strict anoxic conditions. It can grow in a pH range of 4.0 to 7.0, with an optimum pH of 6.0 (Tanner, Miller et al. 1993).

| Species | Opt T °C | Opt pH | Products | Reference |
|---|---|---|---|---|
| **Mesophilic microorganisms** | | | | |
| *Acetobacterium woodi* | 30 | 6.8 | Acetate | Genthner, 1986 |
| *Butyribacterium methylotrophicum* | 37 | 6.0 | Acetate, *n*-butyrate, ethanol, *n*-butanol | Grethlein, 1991 |
| *Butyribacterium methylotrophicum* | 37 | 5.8-6.0 | Acetate, *n*-butyrate, lactate, pyruvate | Shen, 1999 |
| *Clostridium autoethanogenum* | 37 | 5.8-6.0 | Acetate, ethanol | Abrini, 1994 |
| *Clostridium carboxidivorans* | 38 | 6.2 | Acetate, *n*-butyrate, ethanol, *n*-butanol | Liou, 2005 |
| *Clostridium ljungdahlii* | 37 | 6.0 | Acetate, ethanol | Tanner, 1993 |
| *Eubacterium limosum* | 38-39 | 7.0-7.2 | Acetate | Genthner, 1981 |
| *Clostridium ragsdalei* | 37 | 6.3 | Acetate, ethanol | Huhnke, 2008 |
| **Thermophilic microorganisms** | | | | |
| *Moorella thermoacetica* | 55 | 6.5-6.8 | Acetate | Daniel, 1990 |
| *Moorella thermoautotrophica* | 58 | 6.1 | Acetate | Savage, 1987 |

**Table 2-1. Common thermophilic and mesophilic microorganisms**

*Clostridium ljungdahlii* has been widely studied in syngas fermentation for production of ethanol. Most studies have focused on increasing ethanol yield and ethanol to acetate ratio. A study of the effect of yeast extract on the fermentation products showed that lower initial concentrations favor production of ethanol and an increase in the ethanol to acetic acid ratio (Vega, Prieto et al. 1989). Several studies to optimize the growth medium have been performed (Gaddy J.L 2007; Simpson 2010). Omission of yeast extract produced an increase of ethanol concentration up to 20 and 48 g/L in a continuous fermentation system without and with cell

recycling respectively (Klasson, Ackerson et al. 1993; Phillips, Klasson et al. 1993). Different studies showed a negative or no effect of reduced pH on ethanol production and ethanol to acetic acid ratio (Cotter, Chinn et al. 2009; Cotter, Chinn et al. 2009). Recent efforts have been made to produce the biofuel *n*-butanol. *Clostridium ljungdahlii*'s genome was recently sequenced and genes encoding enzymes, such as crotonase and butyryl-CoA dehydrogenase, are not present and the bacterium is therefore not able to synthesize *n*-butanol directly from syngas (Köpke, Held et al. 2010). The researchers introduced a plasmid containing the *Clostridium acetobutylicum n*-butanol synthesis pathway genes and obtained a maximum concentration of 2 mM *n*-butanol in batch cultures of the modified strain.

*Clostridium carboxidivorans*

*Clostridium carboxidivorans* was isolated by incubating sediments of an agricultural lagoon in an acetogen medium at 37°C and initial pH of 5.0, and using CO as a substrate (Liou, Balkwill et al. 2005). The main fermentation products detected were acetic acid, *n*-butyric acid, ethanol, and *n*-butanol (Rajagopalan, P. Datar et al. 2002; Datar, Shenkman et al. 2004), however the bacterium does not contain the genes for acetone synthesis (Bruant, Levesque et al. 2010). It grows in a temperature range of 24 to 42 °C with an optimum range of 37 to 40 °C. The growing pH ranges from 4.4 to 7.6, with an optimum from 5.0 to 7.0 (Liou, Balkwill et al. 2005).

A study of synthetic syngas fermentation in a bubble column showed apparent yields (mol C in products per mol CO consumed) of 0.15, 0.075, and 0.025 for ethanol, *n*-butanol, and acetic acid. *Clostridium ljungdahlii*, which was tested in batch culture with the same substrate, showed

yields of 0.062 and 0.094 for ethanol and acetic acid, respectively (Rajagopalan, P. Datar et al. 2002).

Studies have been conducted to evaluate the ability of *Clostridium carboxidivorans* to grow in biomass generated producer gas to produce alcohols. Cessation of growth, inhibition of $H_2$ uptake, production of alcohols, and alteration of ethanol to acetate ratio were the main findings (Datar, Shenkman et al. 2004; Ahmed, Cateni et al. 2006). Subsequent studies showed that NO is one the compounds responsible for the hydrogenase inhibition and increase of the ethanol production (Ahmed and Lewis 2007).

*Clostridium ragsdalei* P11

A recently isolated *Clostridium* strain P11 or *Clostridium ragsdalei* can produce mainly acetic acid, ethanol, isopropanol, and *n*-butanol using syngas as the substrate (Huhnke 2010; Kundiyana, Huhnke et al. 2010). Intense research has been devoted to this bacterium during recent years. A study of different growth medium ingredients to replace expensive components have shown that cotton seed extract can replace all vitamin and minerals resulting in the prodiction of more ethanol after 15 days of fermentation in bottles (Kundiyana, Huhnke et al. 2010). Use of corn steep liquor to replace yeast extract also resulted in an increasing ethanol production. Growth in the presence of 20 g/L corn steep liquor produced a concentration of 9.6 g/L ethanol whereas 1 g/L yeast extract produced 6.1 g/L after 360 h in a 7.5 L fermenter (Maddipati, Atiyeh et al. 2011).

Other studies of media optimization focused in adding reducing agents to the growth medium to increase the ethanol production. Reducing agents help shuttling electrons from $H_2$ and CO to the

cofactors $NAD^+$ or $NADP^+$ which, when reduced to NADH and NADPH, promote ethanol production (Wilkins and Atiyeh 2011). Addition of dithiothreitol in high concentrations (7.5 – 10 g/L) or sulfide proved to favor ethanol production at increasing concentrations (Hu, Jacobsen et al. 2010; Kubandra Babu 2010).

### *Commercial syngas fermentation*

Fermentation of syngas for biofuel ethanol production has been investigated by several companies, such as Coskata Inc. (http://www.coskata.com), which is building the first commercial facility in Alabama; Ineos BIO (http://www.ineosbio.com) also announced the construction of a facility that will produce 30,000 $m^3$ per year in Indian River, FL, and LanzaTech (http://www.lanzatech.co.nz) in Auckland, New Zealand. Their process is mainly based on fermentation of gases produced by gasification of biomass, coal and municipal residues, and CO containing gases produced in industries, such as oil refining and steel manufacturing. The process consists of gasification of biomass followed by cleaning and cooling, then fermentation in a bioreactor and finally ethanol recovery and dehydration (Munasinghe and Khanal 2010; Abubackar, Veiga et al. 2011).

### *Biochemistry of syngas fermentation*

#### *Wood-Ljungdahl pathway*

Syngas fermenter microorganisms rely on the Wood-Ljungdahl pathway, also called acetyl-CoA pathway for the production of biomass and end products (Henstra, Sipma et al. 2007). The Wood-Ljungdahl pathway is a noncyclic and irreversible pathway, which consists of two

branches: the methyl branch and the carbonyl branch. A $CO_2$ molecule in the methyl branch is reduced to formic acid by formate dehydrogenase using 2 electrons from NADPH (Figure 2-1). Then, formate is condensed with $H_4$folate by a formyl-THF synthetase and form 10-formyl-$H_4$-folate, using one ATP. Then, 10-formyl-$H_4$-folate is converted to 5,10-methenyl-$H_4$-folate by a mathenyl-THF cyclohydrogenase. The latter is then reduced to 5,10-methylene-$H_4$folate by methylene-THF dehydrogenase, using 2 electrons provided by NAD(P)H. 5,10-methylene-H4folate is reduced again to (6S)-5-$CH_3$-$H_4$folate by methylene-THF reductase, which uses 2 electrons from reduced ferredoxin. Finally, a methyl transferase transfers the methyl group to a corrinoid iron sulphur protein (CFeSP) which gives the methyl group to the acetyl CoA synthase/carbon monoxide dehydrogenase (ACS/CODH) (Ragsdale and Pierce 2008) (Figure 2-1). The ACS/CODH condenses the methyl group with a CO and a CoA to form acetyl-CoA (Wood, Ragsdale et al. 1986; Ragsdale 1997). Six electrons and one ATP are consumed in the process (Ragsdale 2008).

*CO dehydrogenase*

CO dehydrogenase is a key enzyme in autotrophic growth of anaerobic syngas fermenter organisms. There are two types of CO dehydrogenases, the Ni-CODH, involved in the Wood-Ljungdahl pathway, and Mo-Cu-Se CODH, present in aerobic bacteria (Ragsdale 2008). The Ni-CODHs are divided in two groups, which are the monofunctional CODH and bifunctional CODH/ACS. Monofunctional CODHs catalyze the reaction presented in Equation 1 and can produce CO for the carbonyl branch of the W-L pathway by reducing $CO_2$ when CO is not present, and can produce $CO_2$ and reduced ferredoxin for the methyl branch when CO is the main substrate. Multifunctional CODH/ACS catalyzes the reaction presented in Equation 1

coupled with the reaction presented in Equation 2 and synthesizes acetyl-CoA (Lindahl 2002; Ragsdale 2008).

$$CO_2 + 2H^+ + 2\bar{e} \rightleftharpoons CO + H_2O \quad \Delta E^{\circ\prime} = -520 \text{ mV} \qquad (1)$$

$$CO + CH3\text{-}CFeSP + CoA \longrightarrow Ac\text{-}CoA + CFeSP \qquad (2)$$

CO oxidation produces reducing power, and electron acceptors used in this reaction are coupled to other cellular process. For instance, NADH is used for $CO_2$ reduction in the methyl branch of the Wood-Ljungdahl pathway (Ragsdale 2002).

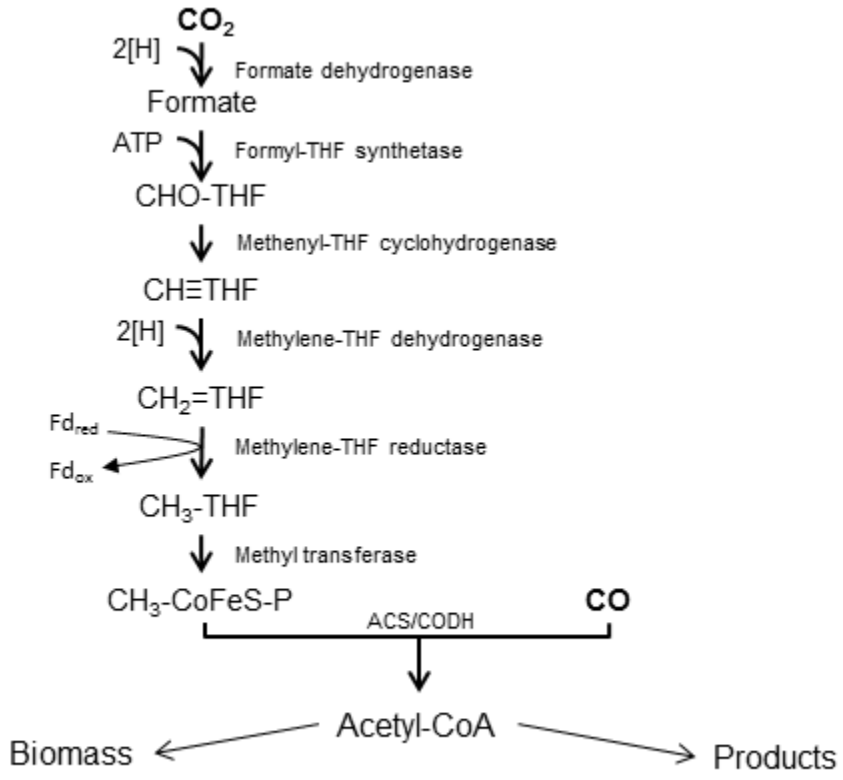



**Figure 2-1. Wood-Ljungdahl pathway**

*Hydrogenases*

Reducing power required for reduction reactions and energy generation when bacteria grow autotrophically on CO and $H_2$, are obtained from CO oxidation by CO dehydrogenase, and $H_2$ *via* hydrogenases. Hydrogenases catalyze the reversible reaction represented in Equation 3 (Adams, Mortenson et al. 1980). They can activate $H_2$ and create reductant for energy production and reduction reactions in the cell, and also can use protons as electron acceptors when no other acceptors are available (Goldman and Mascharak 1995).

$$H_2 \rightleftharpoons 2H^+ + 2e^- \quad (3)$$

There are five types of hydrogenases. Three of them are NiFe hydrogenases, one is Fe only and one contains no metal and catalyzes reaction shown in Equation 4 (Ragsdale 2002). The genome of *Clostridium ljungdahlii* encodes four Fe-only hydrogenases and one NiFe- hydrogenase (Köpke, Held et al. 2010).

$$\text{Methenyltetrahydromethanopterin} + H_2 \rightleftharpoons \text{methylenetetrahydromethanopterin} + H^+ \quad (4)$$

CO has the property to bind the Fe center of hydrogenases producing a reversible inhibition of hydrogenase activity (Tard and Pickett 2009; Matsumoto, Kabe et al. 2011). This affects the $H_2$ consumption during syngas fermentation because the presence of CO in growth media reduces its utilization and the efficiency of CO fixation. Electrons can be obtained from CO *via* CO dehydrogenase or from $H_2$ *via* hydrogenases, and a reduction of hydrogenase activity lead to the utilization of CO as an electron source rather than product formation (Ahmed and Lewis 2007).

*Energy conservation*

Acetogenic bacteria, such as *Acetobacterium woodi*, can use $CO_2$ as an electron acceptor, using the acetyl-CoA pathway to reduce it to acetate. Electrons for $CO_2$ reduction are provided by different compounds, such as sugars, alcohols, molecular hydrogen, or CO (Biegel, Schmidt et al. 2011). Acetogens can also use other alternative electron acceptors, such as nitrate or caffeate (Seifritz, Daniel et al. 1993; Schmidt, Biegel et al. 2009). Some acetogens such as *Clostridium aceticum* and *Moorella thermoacetica*, have some membrane proton translocating electron transport systems, which contain cytochromes, menaquinones, and several oxidoreductases. These organisms create a proton motive force, which is subsequently used for ATP formation in a proton dependent ATPase (Drake, Gossner et al. 2008).

*A. woodi* and other bacteria couple the $CO_2$ reduction with the creation of a $Na^+$ gradient across the membrane. This gradient is used as $Na^+$ motive force by a $Na^+$ dependent ATPase to synthesize ATP (Imkamp and Muller 2002; Muller, Imkamp et al. 2008). Organisms that pump $Na^+$ are dependent of $Na^+$ for growth and also motility can depend of $Na^+$ concentration (Drake, Gossner et al. 2008). Concentrations below 5 mM in *Acetobacterium woodi* cause severe growth defects (Heise, Muller et al. 1989).

*Rnf complex*

Different studies in *Acetobacterium woodii* demonstrated that ferredoxin:$NAD^+$ oxidoreductase (Fno) reaction is coupled to $Na^+$ translocation out of the membrane (Biegel and Muller 2010). Membrane preparations showed that Rnf subunits were present, and using different inhibitors the researchers demonstrated that inhibition of Fno activity also inhibited the $Na^+$ translocation

(Biegel, Schmidt et al. 2009; Biegel and Muller 2010). These observations support the thesis that Rnf complex is responsible for coupling the Fno reaction to the $Na^+$ translocation.

The Rnf complex is a membrane associated electron transport protein complex composed of 6 subunits and there is evidence that each unit is present in a single copy (Biegel, Schmidt et al. 2011). Subunits RnfD, RnfE, and RnfA are proposed to be integral membrane proteins, while subunits RnfG and RnfB are proposed to be membrane associated proteins, and RnfC is proposed to be a soluble protein. Electrons donated by $Fd_{red}$ are proposed to enter the complex by RnfB and travel through RnfE, RnfD, RnfA, RnfG, and finally RnfG, where $NAD^+$ is reduced to NADH. However, the exact function of each subunit is not well known (Biegel, Schmidt et al. 2011).

Sequencing of the *Clostridium ljungdahlii* genome showed a possible new mechanism of energy conservation in acetogens (Köpke, Held et al. 2010). The bacterium possesses all genes that encode the six subunits of the Rnf complex. Also, genes encoding an $H^+$ translocating ATPase were found in *C. ljungdahlii* genome and no $Na^+$-liganding motif was found, suggesting that no $Na^+$ dependent ATPase is present. Experimental data show that there is no effect of $Na^+$ concentrations as low as 1.4 mM in growth, which demonstrates that $Na^+$ is not necessary for growth (Köpke, Held et al. 2010). Köpke et al. propose a third mechanism for energy conservation in acetogens in which the Fno reaction is coupled to a proton translocating Rnf complex that generates a proton gradient, which is used by a proton dependent ATPase for ATP synthesis (Figure 2-2). NADH generated is then used in reduction of $CO_2$ during carboxyl branch of acetyl-CoA pathway. The Rnf complex mechanism and function is still unknown.

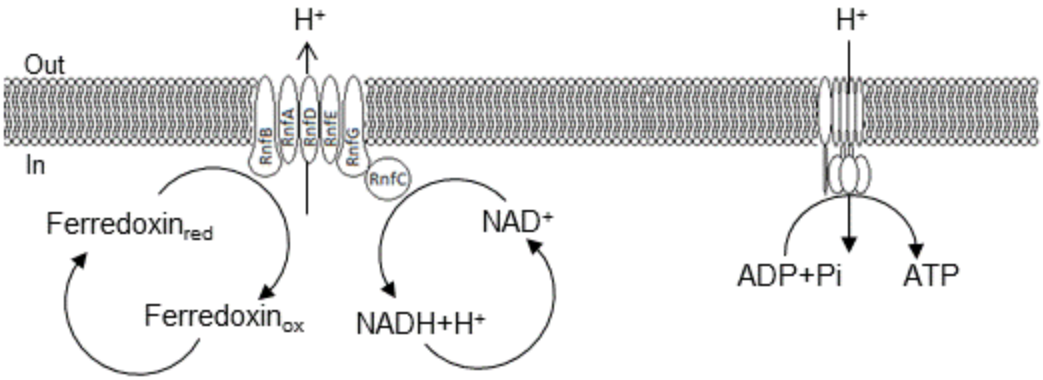



**Figure 2-2. Fno reaction in Rnf complex in *Clostridium ljungdahlii* (Adapted from Köpke et al. 2010)**

*Products synthesis*

The pathways that syngas fermenting organisms use to produce acids and alcohols is presumed to be analogous to the pathways used by *Clostridium acetobutylicum* (Jones and Woods 1986; Tanner 2008). During the acidogenic phase, acetyl-CoA is converted into acetyl-P by phosphotransacetylase and the Co-A group is replaced by a phosphate group. Then, acetyl-P is converted into acetate by acetate kinase and an ATP is produced (Waterson, Castellino et al. 1972; Hartmanis and Gatenbeck 1984). Production of acetate recovers the ATP invested during reduction of $CO_2$ in carboxyl branch of the Wood-Ljungdahl pathway (Figure 2-3).

The *n*-butyrate pathway from acetyl-CoA starts with the transfer of an acetyl group from an acetyl-CoA to another acetyl-CoA by a thiolase (acetyl-CoA acetyltransferase) to form acetoacetyl-CoA (Lee, Park et al. 2008). Then, 3-hydroxybutyryl-CoA dehydrogenase reduces the acetone group in carbon 3 of acetoacetyl-CoA and forms 3-hydroxybutyryl-CoA, which is then dehydrated by a crotonase to form crotonyl-CoA. Crotonyl-CoA is hydrogenated by

butyryl-CoA dehydrogenase and forms butyryl-CoA. A phosphate group replaces the CoA group by a phosphotransbutyrylase to form butyryl-P, which is finally removed by a butyryl kinase to form butyrate and ATP (Valentine and Wolfe 1960; Hartmanis and Gatenbeck 1984).

During solventogenesis, the metabolism is redirected from the acid producing pathways to the solvent producing pathways. Acetyl-CoA is reduced by acetaldehyde dehydrogenase and acetaldehyde is formed. Then, ethanol dehydrogenase reduces acetaldehyde to ethanol. Acetate produced during acidogenesis is activated by acetoacetyl-CoA transferase, which transfers a CoA from acetoacetyl-CoA to an acetate molecule and forms acetyl-CoA and acetoacetate. Acetoacetate is further decarboxylated to form acetone by acetoacetate decarboxylase and acetyl-CoA reduced to ethanol (Hartmanis, Klason et al. 1984; Haggstrom 1985).

A similar process occurs for *n*-butanol synthesis. *n*-Butyrate is activated by acetoacetyl-CoA transferase, which transfers a CoA from acetoacetyl-CoA to *n*-butyrate and forms butyryl-CoA and acetoacetate. A reduction of butyryl-CoA to butyraldehyde and then to *n*-butanol is performed by butyraldehyde dehydrogenase and butanol dehydrogenase respectively. However, a different pathway for *n*-butanol and ethanol synthesis is possible *via* butyrate kinase and phosphotransbutyrylase working in the inverse direction and without the formation of acetone (Hartmanis, Klason et al. 1984; Hüsemann and Papoutsakis 1989). Experiments have been conducted to reduce *n*-butyric acid to *n*-butanol using glucose as source of electrons and energy. For example, *Clostridium saccharoperbutylacetonicum* was grown in continuous fed systems to evaluate the *n*-butyrate reduction to *n*-butanol. When feeding only *n*-butyric acid, the production of *n*-butanol could not be supported, but adding glucose the production of *n*-butanol was higher than glucose fermentation without addition of *n*-butyrate (Tashiro, Takeda et al. 2004; Richter, Qureshi et al. 2012).

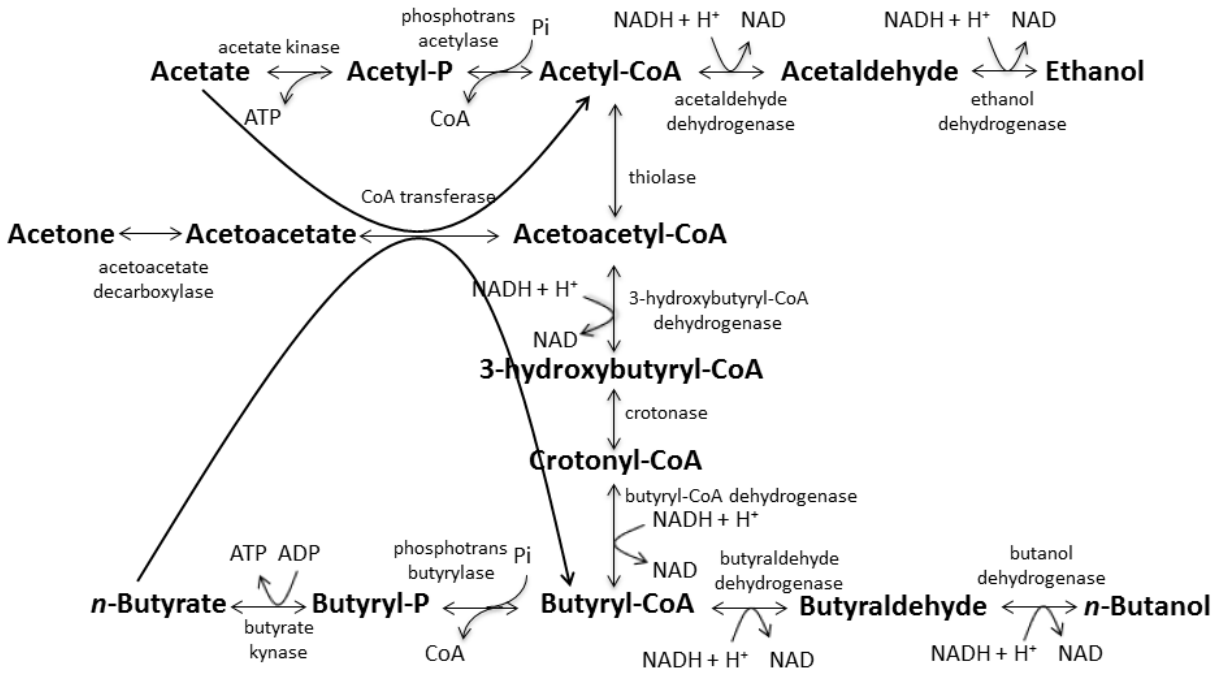



**Figure 2-3. Metabolic pathways of solvent and acid production in *Clostridium acetobutylicum*. (adapted from Lee, 2008)**

*Clostridium carboxidivorans*

Metabolic pathways for acids and alcohols synthesis in *Clostridium carboxidivorans* are very similar to those found in *Clostridium acetobutylicum*. Acetic acid, *n*-butyric acid, ethanol and *n*-butanol are synthesized following the same pathway and catalyzed by enzymes with high levels of identity (Bruant, Levesque et al. 2010). However, no acetoacetate decarboxylase is encoded in the *Clostridium ljungdahlii* genome. Although several CoA transferases are present, it cannot produce acetone. Indirect evidence exists that *Clostridium carboxidivorans*, (similar to *Clostridium acetobutylicum*,) can also convert acids to the corresponding alcohols (Datar, Shenkman et al. 2004).

*Clostridium ljungdahlii*

*Clostridium ljungdahlii* possesses all enzymes for acetic acid and ethanol production from acetyl-CoA. It is also able to consume ethanol using the same pathway. However, its genome also contains an aldehyde oxidoreductase, which catalyzes the reduction of acetate to acetaldehyde, using reduced ferredoxin (Köpke, Held et al. 2010). This reaction saves one ATP in the formation of ethanol but consumes a reducing molecule which can potentially donate electrons to $NAD^+$ in fno reaction, creating proton motive force *via* the Rnf complex. Enzymes that catalyze several steps of the *n*-butyrate and *n*-butanol synthesis, such as crotonase and butyryl-CoA dehydrogenase, are not present. However, *C. ljungdahlii* it has been proven to consume *n*-butanol and to convert it into *n*-butyrate (Köpke, Held et al. 2010), which shows that those enzymes are present and potentially perform the reverse reaction.

# CHAPTER 3

# BIOCATALYTIC REDUCTION OF SHORT CHAIN FATTY ACIDS TO ALCOHOLS *VIA* SYNGAS FERMENTATION


## *Abstract*

Short-chain carboxylic acids generated by various mixed- or pure-culture fermentation processes have been considered valuable precursors for production of bioalcohols. While conversion of carboxylic acids into alcohols is routinely performed *via* catalytic hydrogenation or with strong chemical reducing agents, we explored a biological conversion route. Here, we demonstrate the promising potential of *C. ljungdahlii* as a biocatalyst for conversion of short-chain carboxylic acids into alcohols, using syngas as a source of electrons and energy. Acetic acid, propionic acid, *n*-butyric acid, isobutyric acid, *n*-valeric acid, and *n*-caproic acid were successfully converted into the corresponding alcohols. Furthermore, biomass yields and fermentation stoichiometry from our experimental data enabled us to amend thermodynamic calculations with the goal to evaluate how much of metabolic energy *C. ljungdahlii* can generate during fermentation of carbon monoxide. Average ATP yields of 0.4 ATP per CO consumed were determined. The ratio of protons pumped across the cell membrane *vs*. electrons transferred from ferredoxin to $NAD^+$ *via* the RNF complex is suggested to be 1.0.

## *Introduction*

Limited resources of fossil energy carriers have stimulated the development of renewable liquid transportation fuels. In the USA, Brazil, and Europe a substantial amount of renewable fuel is currently produced as bioethanol *via* fermentation of starch or saccharose obtained from food plants, such as sugarcane, corn, wheat, and sugar beets. In 2011, the total bioethanol production in the USA was 52.6 million $m^3$, while it was 21.1 and 4.4 million $m^3$ in Brazil and Europe, respectively (Renewable Fuels Association http://www.ethanolrfa.org/). This has raised concerns about future fuel *vs*. food competition, and the sustainability of biofuels in general, since the fertilizer- and energy-intensive agriculture, and energy-intensive ethanol separation, reduces the net energy balance (Engelhaupt 2008; Searchinger, Heimlich et al. 2008; Naik, Goud et al. 2010). Therefore, technologies are being developed, which utilize organic materials that are not derived from food crops. Lignocellulose, which is the main component of plants and the most abundant renewable organic material on the planet's surface (Zhang, Ding et al. 2007), is considered the most promising raw material. There are two major routes for conversion of lignocellulose into bioalcohols: the biochemical route (fermentation) and the thermochemical route:

1) Fermentation of lignocellulose is technically feasible, but more challenging than using starch or sugar substrates: A) An extensive pretreatment involving heat, chemicals, and enzymes is required to extract the recalcitrant cellulose and break it down into fermentable sugars (saccharification); B) Toxic chemicals, which can inhibit the consecutive sugar fermentation, are co-produced during pretreatment and have to be removed (Qureshi, Saha et al. 2008; Zhang, Agrawal et al. 2011); and C) The lignin component of lignocellulose cannot be used as a substrate for sugar to bioalcohol fermentation (Munasinghe and Khanal 2010).

2) The thermo chemical route can convert both cellulose and lignin into fuels. It involves thermal pyrolysis into synthesis gas. During gasification, part of the biomass undergoes combustion to provide the energy necessary to break and re-arrange carbon bonds to produce syngas, which is essentially a mix of carbon monoxide, hydrogen gas, and carbon dioxide. The syngas to alcohol conversion can then be performed *via* two different sub-routes in which new carbon bonds are established: A) *via* chemical catalysts in the Fischer-Tropsch process; or B) *via* fermentation, using bacteria as biological catalysts (Tanner 2008). In comparison with the established Fischer-Tropsch process, syngas fermentation is a new technology that comes with inherent challenges: It is associated with limitations in mass transfer of the gaseous substrates, lower productivity, and lower concentrations of end products, resulting in higher energy costs for product purification (Munasinghe and Khanal 2010). On the other hand, fermentation possesses a higher product specificity, operates under lower temperature and pressure, is flexible in the feed gas composition, and the biocatalyst is self-regenerating. For these reasons, we are investigating syngas fermentation as a promising alternative to chemical catalysis processes.

Certain species of homoacetogenic bacteria are able to grow chemolithoautotrophically with syngas and produce organic compounds, which are mainly acetic acid and ethanol (Henstra, Sipma et al. 2007). Of all species, *Clostridium ljungdahlii* has been shown to produce the highest concentrations of ethanol (48 g/L) (Klasson, Ackerson et al. 1993), rendering it a promising biocatalyst for conversion of syngas into ethanol. Although being a dominant alcohol in the biofuel industry, ethanol is not the only one. Production of acetone and *n*-butanol (as chemicals, not as fuels) *via* fermentation has been a viable process during the first half of the 20th century (Jones and Woods 1986). Compared to ethanol, *n*-butanol has properties that render it a more desirable fuel for cars (Lee, Park et al. 2008). Large petrol and pharmaceutical corporations, such

as BP and DuPont, but also numerous innovative startup companies are developing technologies for biobutanol production. These companies are researching the biochemical route for lignocellulose to *n*-butanol fermentation.

Our lab is developing an alternative process for lignocellulose to *n*-butanol conversion that employs both thermochemical and biochemical methods. Briefly, lignocellulose is fermented into *n*-butyric acid using the "carboxylate platform", a fermentation technology with open cultures of microbial consortia for treatment of lignocellulosic biomass and manure that, instead of methane, generates short-chain carboxylic acids, such as *n*-butyric acid, as end products (Agler, Wrenn et al. 2011). Then, *n*-butyric acid is reduced to *n*-butanol, using pure cultures of solventogenic clostridia. This step requires a source of electrons and energy (ATP). In our recently published work this source was glucose (Richter, Qureshi et al. 2012). Monomeric sugars, such as glucose, are a costly feedstock whose use should be minimized when possible, and more economic sources of electrons and energy are desirable. Here, we report results suggesting that syngas, which can be derived from pyrolysis of biomass, has considerable potential to be such a source of electrons and energy. *n*-Butyric acid and *n*-butanol are produced at very low concentrations *via* fermentation of syngas by some bacterial species (Henstra, Sipma et al. 2007), but not by *Clostridium ljungdahlii* due to a lack of specific enzymes in the *n*-butyric acid pathway. Introducing the corresponding genes on a plasmid enables *C. ljungdahlii* to produce *n*-butanol from syngas, albeit the concentrations published, thus far, have been less than 2 milimolar (Köpke, Held et al. 2010). We found, that significantly higher final concentrations of *n*-butanol can be achieved with a wild-type strain of *C. ljungdahlii*, strain ERI-2, when external *n*-butyric acid is supplied in addition to syngas. Furthermore, we found that *C. ljungdahlii* ERI-2 is able to reduce other short-chain carboxylic acids to corresponding alcohols during

consumption of syngas. We demonstrate the promising potential of wild-type *C. ljungdahlii* ERI-2 as a biocatalyst for conversion of carboxylic acids into the corresponding alcohols. Furthermore, an analysis of biomass yield and fermentation stoichiometry enabled us to confirm theoretical calculations regarding the metabolic energy that *C. ljungdahlii* can derive from carbon monoxide fermentation. Our data suggest that the ratio of protons that are pumped across the cell membrane *vs*. electrons that are transferred from ferredoxin to $NAD^+$ *via* the Rnf complex is 1.0.

## *Materials and methods*

All chemicals were purchased from Sigma-Aldrich, St. Louis, MO, unless stated otherwise.

### *Bacterial strain and growth conditions*

All experiments were conducted with *Clostridium ljungdahlii* strain ERI-2 (ATTC 55380), which has been used successfully for syngas to ethanol fermentation (L. 1997; Gaddy 2000; Gaddy J.L 2007). Bacteria were always grown anaerobically at 35°C in ATCC medium # 1754 (Tanner, Miller et al. 1993) with the following modifications: The yeast extract concentration was 0.5 g/L or 0.0 g/L as indicated for each experiment; the syngas atmosphere was a synthetic blend of 60 %[vol/vol] carbon monoxide, 35 % hydrogen, and 5 % carbon dioxide (Airgas East, Ithaca, NY); carboxylic acids (propionic, *n*-butyric, isobutyric, *n*-valeric or *n*-caproic acid) were added to final concentrations of 15 mM before adjustment of the pH to 5.5; the buffer was 150 mM 2-(N-morpholino)ethanesulfonic acid (MES).

*Experimental setup*

Precultures of *Clostridium ljungdahlii* were grown in 250-ml serum bottles containing 20-ml ATCC medium #1756 with 0.5 g/L or 0.0 g/L yeast extract (the latter for inoculation of experiments with no yeast extract) and syngas in the headspace at a pressure of 28 psi.

The experiments to determine *Clostridium ljungdahlii*'s ability to reduce propionic acid, *n*-butyric acid, *n*-valeric acid, *n*-caproic acid, or isobutyric acid (2-methyl propionic acid), to the corresponding alcohols were performed in triplicates in 250-ml rubber-stoppered Pyrex glass bottles (Fisher Scientific, Pittsburgh, PA), which were used as batch reactors, but with constant syngas supply. Three stainless steel needles of different length (21 gauge, Becton Dickinson Franklin Lakes, NJ, and ColeParmer, Vernon Hills, IL) were punched through each rubber stopper as gas supply, vent, and sample port. The reactors were filled with 220-mL ATCC medium # 1756 containing 0.5 g/L yeast extract and one of each carboxylic acids at a concentration of 15 mM, autoclaved, and then equipped with fermentation airlocks (www.winemakingsuperstore.com) filled with 5% sulfuric acid to avoid back-contamination by other microbes or oxygen, and placed in a temperature-controlled recirculating water bath for mechanical agitation using a 15 multi position IKA magnetic stirrer (Cole-Parmer Vernon Hills, IL 60061 USA). Artificial syngas was constantly supplied to up to 12 reactors at a time, at a flow rate of 5 mL/min to each reactor *via* a gassing station with multiple outlets and low-flow brass needle valves (McMaster-Carr, Aurora, OH), sterile Masterflex viton tubing (Cole Parmer, Vernon Hills, IL), 0.22-μm sterile filters (Fisher Scientific), and sterile needles through the rubber stopper (size 6.5, VWR, Radnor, PA) connected to gas diffusion stones (Fisher Scientific). All reactors were inoculated with 2 vol% exponentially growing preculture. The reactors with continuous syngas supply were also used to culture inoculum in medium ATCC

1754 without yeast extract to inoculate batch experiments in medium without yeast extract (see below).

The stoichiometry of substrates and products of syngas fermentation with 15 mM *n*-butyric acid and 0 mM *n*-butyric acid was determined in 1L rubber-stoppered Pyrex glass bottles filled with 68.4 ml modified ATCC medium # 1756 without yeast extract and 1051.6 ml syngas headspace at a pressure of 28 psi. Here, yeast extract was omitted from the growth medium to minimize the interference of organic compounds (amino acids) in the fermentation. Two needles, each connected to a three way valve, were used to take gas or liquid samples from the reactor. Gas pressure was measured using gauge pressure transducers (Model PX26, Omega Engineering, Inc., Stamford, CT) attached to hypodermic needles inserted through a rubber septum on the three way valve of the sampling port. The transducers were connected to a data acquisition (DAQ) system interfaced with a personal computer with LabView® software (National Instruments Co., Austin, TX). Pressure data gathered by the DAQ system were converted to volume of gas at standard temperature and pressure (STP), according to the ideal gas law. Bottles were shaken at 100 RPM in an incubator at 35°C. 1% actively growing pre-culture was used as inoculum. Liquid and gas samples were taken daily. The fermentation was operated for *ca*. 200 h. Each experimental condition was repeated twice (each time in triplicate). Cell density (OD600nm), pH, headspace gas pressure, concentration of carbon monoxide, hydrogen, carbon dioxide in the headspace, and carboxylic acids and solvents in the culture medium, were monitored daily. The experiment with the more consistent growth results was chosen for evaluation. One replicate of each experimental condition was not considered for calculations due to gas leakage resulting in decreased cell growth. Therefore, the results (Table 3-II) were

obtained from duplicate experiments. Carbon balances were calculated for the time period from 25 to 115 h after which maximum cell density had been achieved.

*Analytical procedures*

Cell dry weight concentration in the cultures was determined *via* measurement of the optical density with a Milton Roy Spectronic 1201 spectrophotometer at a wavelength of 600 nm after determining a correlation coefficient of 242 mg dry weight/ (L * OD600nm). The carbon content was calculated using the universal proportion of elements in microbial biomass ($C_5H_7O_2N$) (Drapcho 2008). A gas chromatography system (HP 5890, Hewlett Packard, Palo Alto, CA), which was equipped with a 7673 autoinjector and flame ionization detector was used for the quantification of carboxylic acids and alcohols. GC columns were purchased from Sigma-Aldrich. Gas samples were analyzed with two Gow Mac gas chromatographs series 580 (Bethlehem, PA) that were equipped with a thermal conductivity detector. For hydrogen quantification, a 4.5 m Supelco 60/80 Carboxen 1000 column and Nitrogen as carrier gas were used. For carbon dioxide and carbon monoxide, the gas chromatograph was equipped with a 1.8 m Supelco 80/100 Hayesep Q column, and helium was the carrier gas.

***Results and Discussion***

*Conversion of carboxylic acids into alcohols*

*Clostridium ljungdahlii* strain ERI-2 was tested for its ability to reduce short-chain carboxylic acids into the respective corresponding alcohol during continuous syngas supply as described in the methods section. Six conditions were tested: A) no carboxylic acid added (negative control); B) 15 mM propionic acid; C) 15 mM *n*-butyric acid; D) 15 mM *n*-valeric acid; E) 15 mM *n*-caproic acid; and F) 15 mM isobutyric acid.

Growth was observed for all 6 conditions (Figure 3-1). Cell yields and growth rates were similar for the negative control and the conditions with propionic acid and *n*-butyric acid. There was a tendency for the growth parameters to decrease with increasing carbon chain length of the added carboxylic acids (Table 3-I), indicating an increased toxicity of the longer compared to shorter chain carboxylic acids. Moving the carboxyl group from primary into secondary position (iso-butyric acid *vs*. *n*-butyric acid) also resulted in lower growth rates. The pH value in all experiments decreased from 5.5 to *ca*. 4.0 (Table 3-I), with a final slight pH increase during the death phase of the cultures. The lowest final pH value was reached when no external carboxylic acid had been added (Table 3-I, Figure 3-1). Acetic acid and ethanol were produced during syngas fermentation and in the presence of each of the tested carboxylic acids (Figure 3-1). The ratio of acetic acid *vs*. ethanol produced was always close to 1 (Table 3-I).

All added carboxylic acids were consumed by *C. ljungdahlii*. The affinity of the conversion pathway to linear carboxylic acids seems to be higher than to branched chain carboxylic acids, which is reflected in the lower concentration of *n*-butyric acid compared to iso-butyric acid at the end of the fermentation. For each type of carboxylic acid, the apparent efficiency of conversion

into the corresponding alcohol was calculated: 92 % of the carbon amount of consumed propionic acid was found in *n*-propanol, 68 % carbon from *n*-butyric acid was recovered in *n*-butanol, 52 % carbon from *n*-valeric acid in *n*-pentanol, and 46 % C from *n*-caproic acid in *n*-hexanol. The lowest carbon recovery of 42 % was found for conversion of iso-butyric acid to iso-butanol. We hypothesized that the low carbon recovery values had been caused by gas-stripping of the produced alcohols *via* the continuous flow of syngas, and not by cell metabolism. This assumption is supported by the results of the following experiment in which gas stripping was avoided in a *n*-butyric acid to *n*-butanol conversion experiment with a defined amount of syngas in a pressurized reactor. Thus, for a pilot or full scale syngas fermentation system product recovery from the off gases is necessary.

*Stoichiometry of syngas fermentation without and with* n-*butyric acid*

For formation of acetic acid or ethanol from carbon monoxide by syngas fermenting bacteria, the following theoretical stoichiometries are often reported (Heiskanen, Virkajärvi et al. 2007):

$$4\ CO + 2\ H_2O \rightarrow CH_3COOH + 2\ CO_2 \quad (1)$$

$$6\ CO + 3\ H_2O \rightarrow C_2H_5OH + 4\ CO_2 \quad (2)$$

When carbon dioxide and hydrogen are the substrates, the theoretical fermentation equations are:

$$2\ CO_2 + 4\ H_2 \rightarrow CH_3COOH + 2\ H_2O \quad (3)$$

$$2\ CO_2 + 6\ H_2 \rightarrow C_2H_5OH + 3\ H_2O \quad (4)$$

In our study, a mix of carbon monoxide (60%), carbon dioxide (5%), and hydrogen (35%) was supplied, and mixtures of acetic acid and ethanol were produced; consequently, the empirical

fermentation balance was expected to be an intermediate of the previous theoretical equations, complicated further by the addition of *n*-butyric acid and by *n*-butanol production.

Batch experiments with a defined amount of syngas in the headspace were conducted in closed systems as described in the methods section to obtain accurate stoichiometries for syngas fermentation with 15 mM *n*-butyric acid or without *n*-butyric acid. A final pH of 5.1 was obtained in the cultures without *n*-butyric acid. In the setup with 15 mM *n*-butyric acid, the pH decrease was mitigated by the consumption of *n*-butyric acid, resulting in a final pH of 5.3. The empirical fermentation stoichiometries from this study for each condition were normalized to one mol ethanol, which was the major fermentation end product:

$$7.1\ CO + 1.0\ H_2 \rightarrow 1.0\ \text{ethanol} + 0.1\ \text{acetic acid} + 5.0\ CO_2 + 0.4\ \text{cell carbon} \quad (5)$$

$$6.7\ CO + 2.0\ H_2 + 0.1\ \textit{n}\text{-butyric acid} \rightarrow 1.0\ \text{ethanol} + 0.1\ \text{acetic acid} + 4.7\ CO_2 + 0.1\ \textit{n}\text{-butanol} + 0.4\ \text{cell carbon} \quad (6)$$

In the setup with *n*-butyric acid, the relative hydrogen consumption and alcohol production are higher than in the negative control, because solventogenesis (i.e., production of alcohols) seems to be slightly stimulated. The values of carbon recovery (Table 3-II), which were calculated based on product formation *vs*. consumption of substrates, were calculated as 100.2%, demonstrating that all substrates and products had been considered. This result supports the idea that the high losses of carbon in the previous carboxylic acid conversion experiments had been caused by gas stripping of the alcohol products. Gas stripping preferably removes alcohols over carboxylic acids, since alcohols are less polar, less soluble in water, more volatile, and have

higher vapor pressures than the corresponding carboxylic acids. This suggests a possibility for gas stripping as a method for product removal in an industrial carboxylic acid to alcohol conversion plant (Richter, Qureshi et al. 2012).

No *n*-butyric acid was metabolized by *Clostridium ljungdahlii* in a pathway other than conversion to *n*-butanol, as shown by the conversion efficiency. The maximum *n*-butanol concentration in the *n*-butyric acid condition was 13.3 mM. This is considerably higher than the 2 mM that have been published for *n*-butanol synthesis from syngas with a genetically modified *C. ljungdahlii* (Köpke, Held et al. 2010), and can be further improved by optimization of the feeding-rate of *n*-butyric acid. Obviously, *n*-butanol production has also a large potential to be improved further by genetic engineering.

*Energetic calculations, flux of carbon, electrons and protons, and ATP formation*

We need to know the amounts of energy that are contained, lost, and conserved in the substrates and products for evaluation of the energetic efficiency of the fermentation process. Therefore, we calculated the energy balance of the syngas fermentation without and with *n*-butyric acid to *n*-butanol conversion. Values for combustion energy contained in substrates and products were calculated from the standard Gibbs free energies of formation of all compounds (Thauer, Jungermann et al. 1977; Lide 2008), and normalized to 1 mol of ethanol formed (Table 3-II). Around 75 % of the energy contained in the substrate gases and *n*-butyric acid was conserved in the fermentation products. 64 % or 55 % of substrate-energy was conserved in ethanol, and about 9% in *n*-butanol. It should be possible to improve the energy conservation in *n*-butanol at the expense of ethanol by optimizing the *n*-butyric acid feeding method to divert electron flow to butyryl-CoA instead to acetyl-CoA.

In the following, we discuss how much metabolic energy (ATP) is generated during syngas fermentation without and with *n*-butyric acid to contribute insight regarding the mechanism of energy conservation. Bacteria have to spend energy for synthesizing biomass from carbon and other macronutrient elements (Madigan 1997). The energy necessary to synthesize biomass is inversely proportional to the carbon content of the carbon source (Buckel 1999). For synthesis of 10g DW of biomass, approximately 1 mol ATP is necessary when the carbon source contains 6 carbon atoms per molecule, as is the case with glucose. Cells growing on substrates, such as CO or $CO_2$, need approximately 6 mol ATP to synthesize 10 g DW of biomass (Buckel 1999). During acetic acid production from carbon monoxide *via* the Wood-Ljungdahl metabolic pathway, no net ATP is spent or conserved *via* substrate level phosphorylation: One ATP is consumed in the methyl branch, and one ATP is conserved in the acetate kinase reaction during acetic acid formation from acetyl-CoA (Kopke, Mihalcea et al. 2011). For ethanol production from carbon monoxide, one net ATP is spent during substrate level phosphorylation per molecule ethanol produced. Reduction of *n*-butyric acid to *n*-butanol uses 1 ATP for *n*-butyric acid activation *via* the butyrate kinase and phosphotransbutyrylase reaction per mol *n*-butyric acid reduced. It is, therefore, clear that ATP formation during energy metabolism has to occur *via* a mechanism other than substrate level phosphorylation. A recently suggested mechanism is formation of a transmembrane proton potential *via* the so-called Rnf complex that pumps protons using energy derived from electron transfer from reduced ferredoxin to $NAD^+$ (Köpke, Held et al. 2010; Biegel, Schmidt et al. 2011). The membrane potential is then used to generate ATP *via* a membrane-bound ATP-synthase.

The fermentation balance was used for calculation of the amount of ATP spent for carbon fixation in biomass and spent for production of alcohols (Table 3-II). This ATP was then

normalized by mol CO consumed to generate a model for metabolic carbon and electron flux through the Wood-Ljungdahl pathway for both negative control and *n*-butyric acid conditions (Figure 3-3). The amounts of electrons available *via* ferredoxin were calculated based on the knowledge that all electrons derived from oxidation of carbon monoxide and hydrogen are first transferred to ferredoxin *via* CO-dehydrogenase and hydrogenase, respectively, before they proceed to reduce metabolic intermediates with more positive redox-potentials (Ragsdale and Pierce 2008; Köpke, Held et al. 2010). Some of the reduced ferredoxin ($Fd_{red}$) is required to reduce methylenetetrahydrofolate to methyltetrahydrofolate in the methyl branch of the Wood-Ljungdahl pathway (Ragsdale and Pierce 2008). The remaining part of the $Fd_{red}$ is then available to the Rnf complex for generation of a transmembrane proton motive force and consecutive ATP synthesis *via* a membrane-bound ATP synthase.

No experimental results are reported in the literature about the stoichiometry of protons pumped *vs*. electrons transferred from $Fd_{red}$ to $NAD^+$ *via* Rnf complex. The redox couples $Fd_{red}/Fd_{ox}$ (-420 mV) and $NADH_2/NAD$ (-320 mV) have a redox potential difference of 100 mV, which is equivalent to an energy difference of -20 kJ/mol (Muller, Imkamp et al. 2008). Müller *et. al*. speculated that this energy difference would be enough to pump one cation across the cell membrane in *Acetobacterium woodii*, resulting in a ratio of 0.5 protons pumped per electron transferred from $Fd_{red}$ to $NAD^+$. For their calculation, they assumed an electrochemical ion potential of -200 mV for *A. woodii*, but did not state from which source this value was obtained. For *C. ljungdahlii*, no actual data are available, but we believe that it is reasonable to assume that its electrochemical ion potential is close to that of *Clostridium acetobutylicum*, which is a related bacterium for which actual data are available for the transmembrane pH gradient and the electrical potential for different pH values in the growth medium (Huang, Gibbins et al. 1985).

Using these data, we calculated the energy ΔG required for translocation of 1 mol protons at external pH values of 6.5 and 4.5, using equation (7),

$$\Delta G = 2.303 * RT * \log(c2/c1) + ZF\,\Delta V \qquad (7)$$

where R is the ideal gas constant ($8.315*10^{-3}$ kJ*mol$^{-1}$*K$^{-1}$), T is temperature (308 K), log(c2/c1) is ΔpH (0.2 for external pH of 6.5, and 1.5 for external pH of 4.5), Z is charge of the protons (+1), F is Faraday constant (96.49 kJ*mol$^{-1}$*V$^{-1}$) and ΔV is electric membrane potential in Volt of the destination side of the proton (-0.09 V and 0.00 V for an external pH of 6.5 and 4.6, respectively). ΔG values calculated were 9.9 kJ/mol for an external pH of 6.5 and 8.90 kJ/mol for external pH of 4.5. These values suggest that -20 kJ energy is sufficient to pump 2 protons across the membrane in *Clostridium acetobutylicum*, if a mechanism exists, and likely in *C. ljungdahlii* as well. This would result in a stoichiometry of 2 protons pumped per ferredoxin oxidized *via* the Rnf complex. Finally, we have to consider, that synthesis of 1 ATP *via* ATP synthase requires between 3 and 4 protons translocated back into the cytoplasm (Berg 2002; Meier, Ferguson et al. 2006; Ferguson 2010). Based on this consideration and the above energetic calculation, our model suggests that a total of 0.39 to 0.52 mol ATP was produced per mol CO consumed in the negative control, and between 0.47 to 0.63 mol ATP per mol CO in *n*-butyric acid condition (Figure 3-3). These values are very well in agreement with the calculated quantities of ATP required for the synthesis of the determined amounts of biomass and products (Figure 3-3). In the negative control, per mol carbon monoxide oxidized, a total of 0.49 ATP are necessary for the synthesis of biomass and ethanol, whereas in the *n*-butyric acid condition a total of 0.52 ATP are necessary for biomass and ethanol synthesis plus *n*-butyric acid reduction to *n*-butanol. Consequently, we suggest that the stoichiometry of protons pumped per electron transferred from $Fd_{red}$ to $NAD^+$ *via* the Rnf complex is 1 proton/electron.

***Conclusions***

*Clostridium ljungdahlii*, although lacking 2 enzymes essential for synthesis of *n*-butyric acid, can produce significant amounts of *n*-butanol or other alcohols during syngas fermentation when external *n*-butyric acid or other carboxylic acids are provided. The alcohol product has a carbon chain length identical with its carboxyl substrate. The enzymatic machinery for the conversion of the carboxyl into an alcohol group possesses a broad specificity for carboxylic acids of different carbon chain length. However, linear carboxylic acids seem to be favored over their iso-forms. *C. ljungdahlii* ERI-2 (and potentially other syngas fermenting microbes) is, therefore, an ideal catalyst for conversion of, for example, *n*-butyric acid into *n*-butanol, due to its high substrate and product specificity, which is important for industrial applications. Addition of *n*-butyric acid to the growth medium seems to stimulate hydrogen utilization and solventogenesis in *C. ljungdahlii*. This finding also bears potential for application in the biofuel industry. Our experimental results support thermodynamic calculations that the stoichiometry of protons pumped per electrons transferred from ferredoxin to $NAD^+$ *via* the Rnf complex is 1.0. Therefore, hydrogen alone, *via* hydrogenase, Rnf complex and ATP synthase, can theoretically provide the required stoichiometry of 1 ATP and 2 $NADH_2$ to activate and reduce *n*-butyric acid to *n*-butanol. Replacing carbon monoxide and carbon dioxide by hydrogen for conversion of *n*-butyric acid into *n*-butanol could potentially eliminate the formation of byproducts, such as acetic acid and ethanol, in cultures of non-growing cells, and could also minimize the production of carbon dioxide. This idea, of course, still requires experimental proof and would also render the substrate gas more expensive.

***Acknowledgements***

This work was possible thanks to the US Department of Transportation which provided funding to support it under contract to the Northeast Sun Grant Initiative at Cornell University # US DOT Assistance # DTOS59-07-G-00052. Funding was also provided by Yossie Hollander and the Foundation des Fondateurs.

## *Tables and figures*

Table 3-I

| condition | no. of carbon atoms in carboxylic acid | max. opt. density at 600 nm | growth rate ($h^{-1}$) | final pH | consumed carboxylic acid (mM) | produced at end of experiment (mM) | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | alcohol from carboxylic acid | final acetic acid | final ethanol |
| **No carboxylic acid** | NA | 2.73 ± 0.24 | 0.094 ± 0.001 | 3.95 ± 0.04 | NA | NA | 123.27 ± 10.52 | 135.53 ± 20.10 |
| **propionic acid** | 3 | 2.69 ± 0.19 | 0.084 ± 0.012 | 4.11 ± 0.11 | 11.26 ± 1.03 | 10.44 ± 1.69 *n*-propanol | 138.02 ± 22.99 | 169.05 ± 61.65 |
| ***n*-butyric acid** | 4 | 2.71 ± 0.39 | 0.094 ± 0.004 | 4.10 ± 0.11 | 12.13 ± 1.79 | 8.27 ± 1.75 *n*-butanol | 126.36 ± 20.85 | 129.87 ± 43.23 |
| ***n*-valeric acid** | 5 | 2.01 ± 0.04 | 0.077 ± 0.007 | 4.16 ± 0.03 | 10.88 ± 1.07 | 5.63 ± 0.92 *n*-pentanol | 100.48 ± 5.61 | 109.58 ± 25.88 |
| ***n*-caproic acid** | 6 | 1.47 ± 0.17 | 0.049 ± 0.003 | 4.26 ± 0.07 | 11.11 ± 1.18 | 5.11 ± 0.68 *n*-hexanol | 101.10 ± 14.31 | 102.58 ± 39.98 |
| **iso-butyric acid** | 4 | 2.50 ± 0.31 | 0.077 ± 0.008 | 4.08 ± 0.04 | 7.47 ± 4.03 | 3.14 ± 1.89 iso-butanol | 112.90 ± 12.99 | 137.76 ± 46.10 |

**Table 2-I: Parameters for syngas fermentation by *Clostridium ljungdahlii* ERI-2 in medium amended with 15 mM carboxylic acid of different carbon chain length. Values were obtained from triplicate batch cultures with a constant supply of syngas.**

Table 3-II

| | no *n*-butyric acid | + *n*-butyric acid |
|---|---|---|
| **maximum $OD_{600}$** | 1.51 ± 0.12 | 1.34 ± 0.21 |
| **cell mass DW (mg)** | 18.93 ± 1.85 | 16.65 ± 2.99 |
| **carbon fixed in cell mass DW (mmol)** | 1.58 ± 0.15 | 1.39 ± 0.25 |
| **pH after 115 h** | 5.08 ± 0.02 | 5.31 ± 0.04 |
| **ATP for biomass synthesis (mmol)** | 11.36 ± 1.11 | 9.99 ± 1.79 |
| **carbon monoxide consumed (mmol)** | 30.11 ± 2.09 | 25.90 ± 3.13 |
| **hydrogen consumed (mmol)** | 4.19 ± 0.89 | 7.81 ± 3.90 |
| ***n*-butyric acid consumed (mmol)** | NA | 0.31 ± 0.05 |
| ***n*-butyric acid consumed (%)** | NA | 76.3 ± 0.30 |
| **carbon dioxide produced (mmol)** | 21.51 ± 2.16 | 18.19 ± 2.29 |
| **acetic acid produced (mmol)** | 0.23 ± 0.01 | 0.22 ± 0.07 |
| **ethanol produced (mmol)** | 4.25 ± 0.42 | 3.84 ± 0.52 |
| ***n*-butanol produced (mmol)** | NA | 0.31 ± 0.04 |
| **ATP for alcohol production (mmol)** | 4.25 ± 0.42 | 4.15 ± 0.57 |
| **Overall Carbon recovery (%)** | 106.3 ± 3.1 | 106.7 ± 1.4 |
| **Combustion energy (kJ) in all substrates** | 2054 | 2387 |
| **Combustion energy (kJ) in all products** | 1545 | 1756 |
| **Combustion energy (kJ) in ethanol** | 1324 | 1324 |
| **Combustion energy (kJ) in butanol** | NA | 207 |

**Table 3-II: Fermentation balances of batch syngas fermentations without and with 15 mM *n*-butyric acid added. Abbreviations: OD600 = optical density at 600 nm; DW = dry weight; values given in mmol are the total amounts of substrates and products or ATP consumed. Percent carbon recovery considers all substrates, products and carbon fixed in biomass. Energy data (kJ) are normalized to 1 mol ethanol produced.**

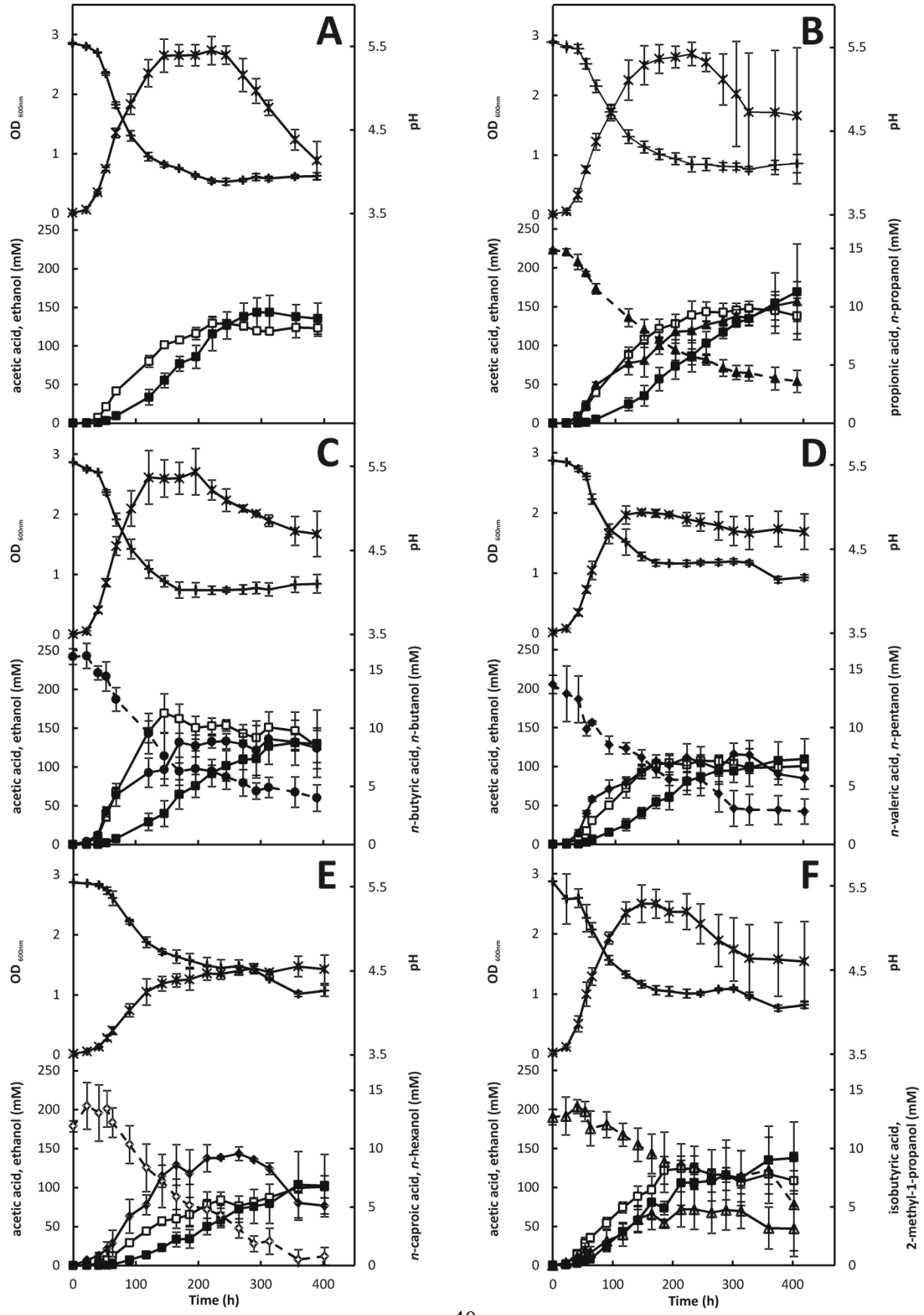

A
B
C
D
E
F
OD 600nm
pH
acetic acid, ethanol (mM)
propionic acid, n-propanol (mM)
n-butyric acid, n-butanol (mM)
n-valeric acid, n-pentanol (mM)
n-caproic acid, n-hexanol (mM)
isobutyric acid, 2-methyl-1-propanol (mM)
Time (h)

**Figure 3-1: Carboxylic acid reduction experiments with *Clostridium ljungdahlii* ERI-2 in medium amended with 15-mM carboxylic acid of different carbon chain length. Values were obtained from triplicate batch cultures with a constant supply of syngas. A) no acids, B) 15 mM propionic acid, C) 15 mM *n*-butyric acid, D) 15 mM *n*-valeric acid, E) 15 mM caproic acid, and F) 15 mM isobutyric acid. OD(600 nm) (X), pH (+), concentrations of acetic acid (□), propionic acid (▲, dashed line), *n*-butyric acid (●, dashed line), *n*-valeric acid (♦, dashed line), *n*-caproic acid (◊, dashed line), isobutyric acid (Δ, dashed line), ethanol (■), propanol (▲), *n*-butanol (●), *n*-pentanol (♦), *n*-hexanol (◊), isobutanol (2-methy-1-propanol) (Δ). Error bars indicate one standard deviation.**

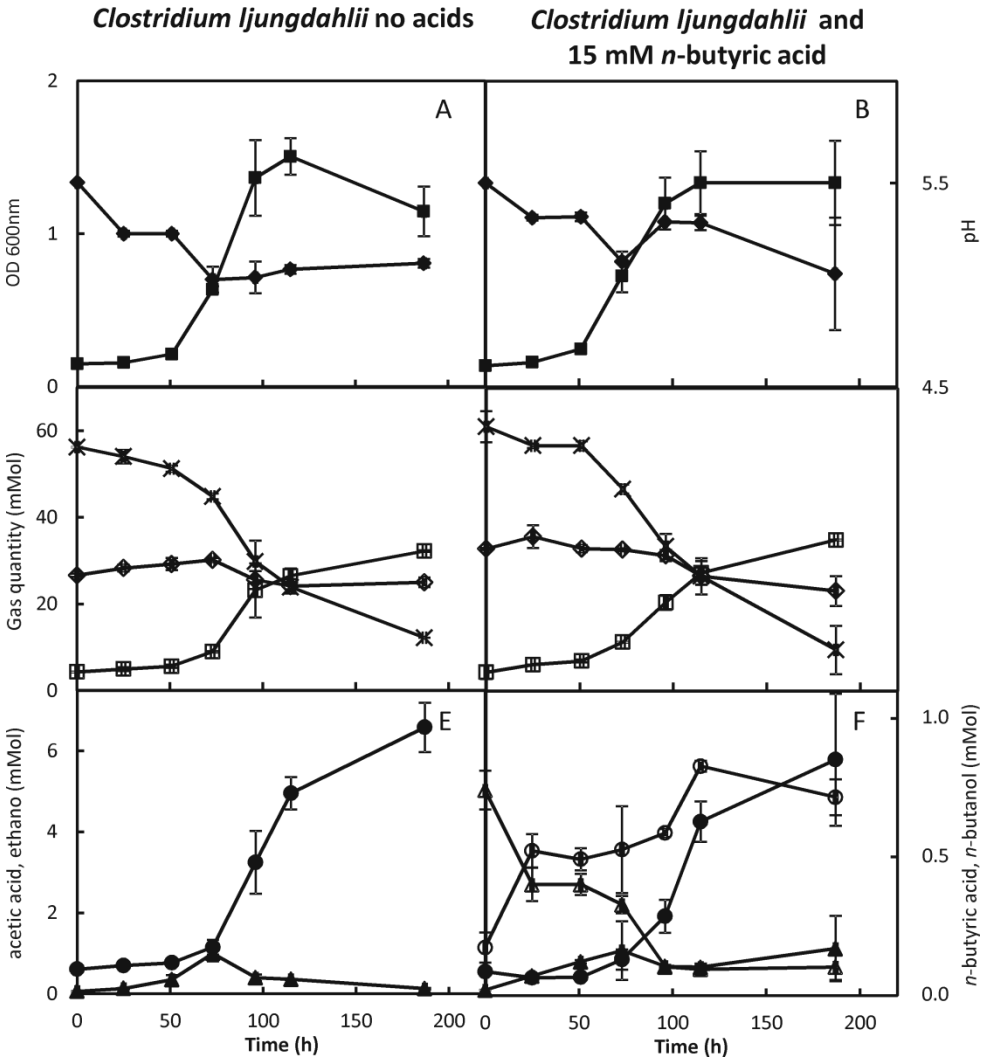
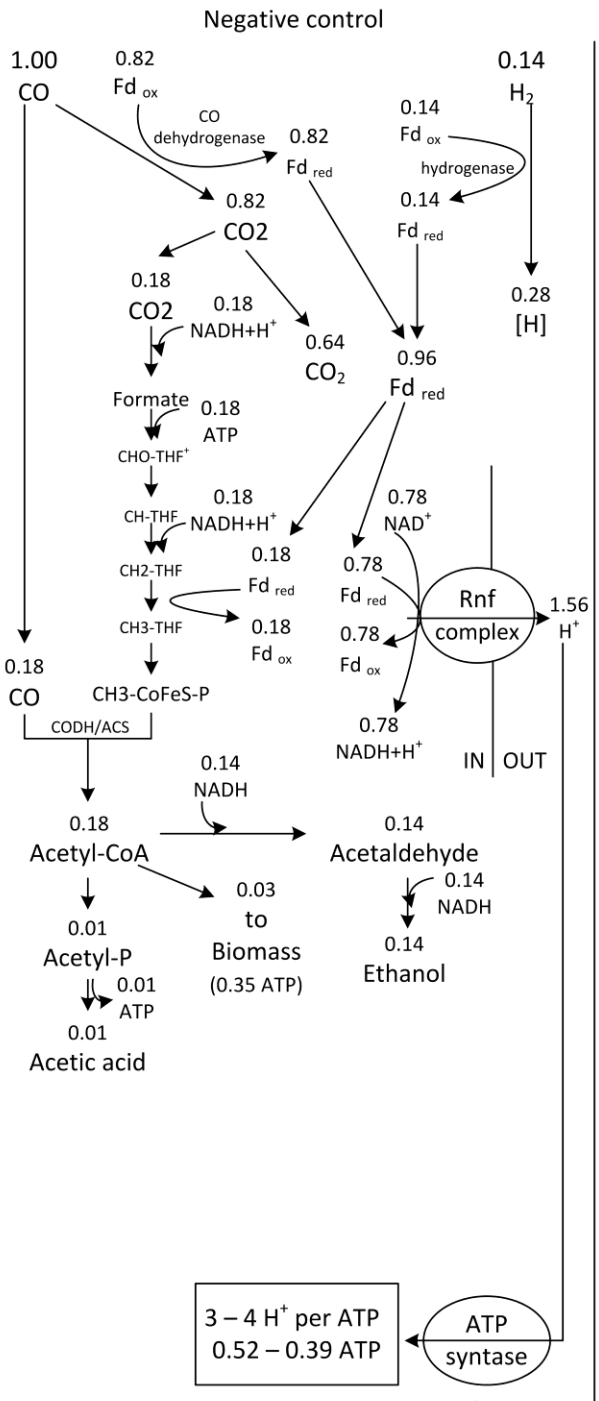


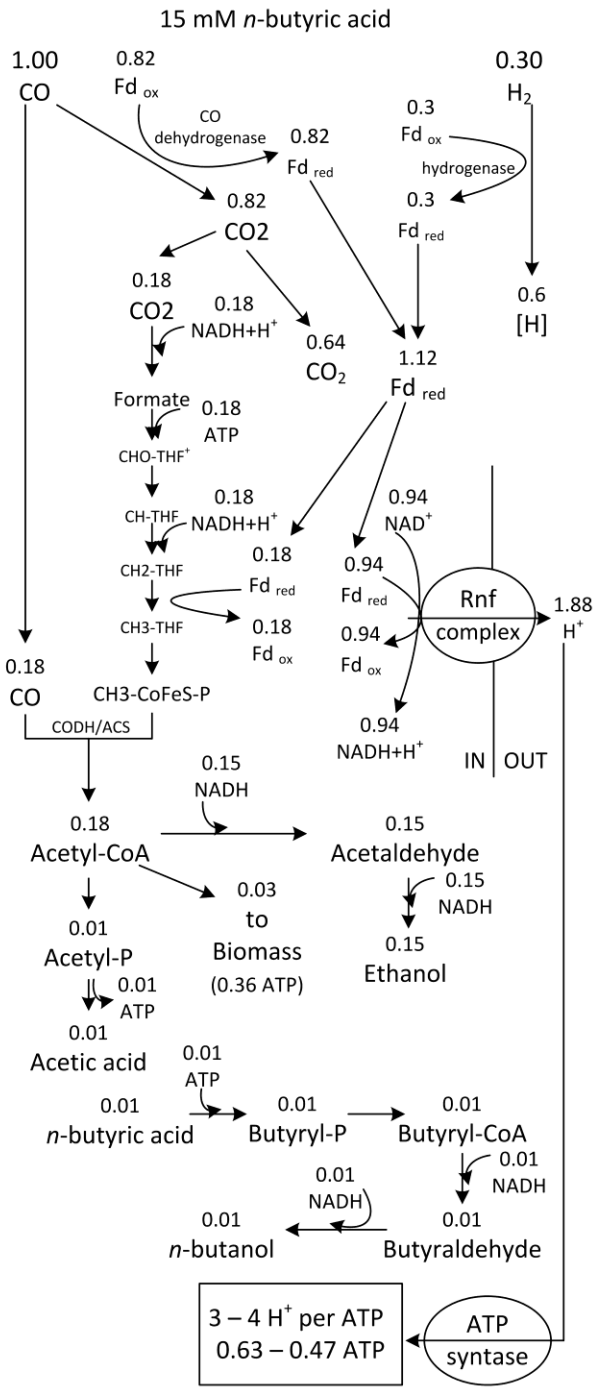

**Figure 3-2: Batch syngas fermentation with *Clostridium ljungdahlii* ERI-2 in 1050-ml reactors without and with 15 mM *n*-butyric acid. OD600nm (■), pH (♦), total amount of acetic acid (▲), *n*-butyric acid (Δ), ethanol (●), *n*-butanol (○), quantities of CO (×), $H^2$ (◊), and $CO^2$ (□). Error bars indicate one standard deviation.**




Thermodynamic calcutation based on 1 $H^+$ pumped/electron transferred via Rnf complex

**ATP spent for**

| | |
|---|---|
| Ethanol synthesis | 0.14 ATP |
| Biomass synthesis | 0.35 ATP |
| Total | 0.49 ATP |




Thermodynamic calcutation based on 1 $H^+$ pumped/electron transferred via Rnf complex

**ATP spent for**

| | |
|---|---|
| Ethanol synthesis | 0.15 ATP |
| *n*-butyric acid conversion | 0.01 ATP |
| Biomass synthesis | 0.36 ATP |
| Total | 0.52 ATP |

**Figure 3: Model for flux of carbon and electrons, and energy balance for negative control and 15 mM *n*-butyric acid. Values are normalized to mol CO consumed. Energy required for biomass formation is estimated based on 6 ATP per 10 grams dry weight biomass synthesized from carbon monoxide. All data are obtained during the growth phase.**

# CHAPTER 4

# GENERAL CONCLUSIONS AND FUTURE DIRECTIONS

## *General conclusions*

This work tested the ability of *Clostridium ljungdahlii* to convert organic acids into the corresponding alcohols using syngas as a source of energy and reducing power. Also, stoichiometry of the conversion was investigated and carbon, electrons and energy flux was studied. The general conclusions are presented in relation to the hypotheses formulated.

*Hypothesis 1: Clostridium ljungdahlii is able to convert organic acids of different molecular weight into the corresponding alcohols using syngas as source of energy and reducing power*.

Results obtained from cultures of *Clostridium ljungdahlii* tested in presence of propionic acid, *n*-butyric acid, *n*-valeric acid, *n*-caproaic acid, and isobutyric acid using syngas as a source of energy and reducing power showed that the bacterium has the enzymatic machinery to reduce carboxylic acids to the corresponding alcohols. Even though it lacks of some enzymes necessary for carbon chain elongation, it can produce alcohols with a carbon chain length longer than two carbons when the corresponding carboxylic acid is provided. Alcohols produced in presence of carboxylic acids contain the same number of carbons of the carboxylic acid supplied in the growth medium, showing that there is no transformation of the carbon chain, but only the acid group.

The enzymatic machinery has a broad specificity for carboxylic group, being able to reduce carboxylic acids of different carbon chain length. However it seems to favor primary acids over their isoforms. Addition of carboxylic acids to the growth medium stimulates $H_2$ consumption

and production of alcohols. This stimulation has potential application in the industry to increase the utilization of the $H_2$ present in the syngas and to obtain more specific fermentation products. We hypothesize that CO could be replaced by $H_2$ in non-growing cultures to convert carboxylic acids into the corresponding alcohols preventing the production of other byproducts.

*Hypothesis 2: Rnf complex in Clostridium ljungdahlii is able to pump 2 $H^+$ out of the membrane per $Fd_{red}$:$NAD^+$ oxidoreduction reaction*.

Our energy balance supports the hypothesis of two protons are pumped out of the cell membrane by Rnf complex per ferredoxin:$NAD^+$ oxidoreduction reaction. No data has been published before related to the function of the Rnf complex.

***Future directions***

Several questions arise from our results and suggest areas for new research. It was proven that *Clostridium ljungdahlii* can convert carboxylic acids to the corresponding alcohols using syngas as a source of energy and electrons but the gene expression and its regulation still remain to be understood. A proteomic study can give insight about the proteins synthesized under the presence of different carboxylic acids. Then, knocking out specific genes such as ethanol production pathway can improve the efficiency of carboxylic acids reduction by driving all electrons to this pathway.

As we suggested above, higher utilization of hydrogen in presence of carboxylic acids can be studied by supplying high $H_2$ synthetic syngas or pure $H_2$ in no growing cultures of *Clostridium ljungdahlii*, but this still has to be tested.

# APPENDIX

### *Experimental setup for continuous syngas fermentation experiments*

The system was designed to be able to run reproducible experiments with several different conditions at the same time. It consists of a water bath placed on a multi position magnetic stirrer in order to provide consistent temperature control and agitation. The syngas was conducted from a gas tank placed into a safety cabinet using 1/4 in stainless steel tubbing, which was connected to two manifolds in a row with six outlets each manifold, totalizing twelve outlets. Each outlet consists of a hose barbed connector, connected to a 1/8 in neoprene tubbing with a luer-lock connector at the end.

Reactors were made of 250-ml capacity media bottles. A rubber stopper was used to close the top opening. The stopper was held with an open cap and a washer. A needle through the stopper was used as the syngas inlet and inside the bottle was connected to a neoprene tube with a sparging stone attached at the end. A second needle was used as the exhaust and was connected to a gas trap in order to prevent oxygen to come in. Finally, a 6 in needle was used to take liquid samples. This needle was closed with a luer-lock cap when not sampling (Figure A-1).

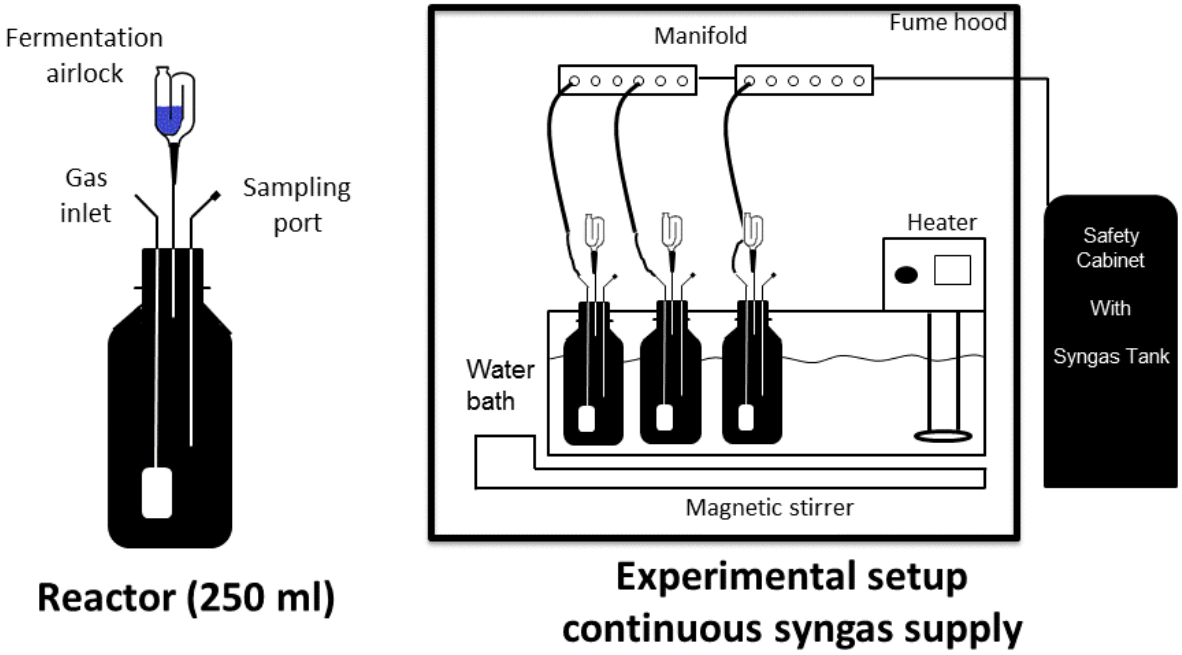



**Figure A-1. Experimental setup for continuous syngas experiments**

***Experimental setup for batch syngas experiments***

This experimental setup was designed to run syngas fermentation experiments in batch cultures with limited amount of syngas. The reactors are gastight and allow headspace pressure measurement as well as gas sampling for gas composition analysis. Liquid samples can also be taken for cell density, pH, and product concentration measurement. The reactors consist of a 1120 ml bottle with a rubber stopper in the top opening secured by a metal washer and a screw cap ring in order to keep it pressurized. One needle punched through the rubber stopper was used to take liquid samples. The needle was connected to a neoprene tubing in order to reach the bottom of the reactor for sampling. The outer part of the needle was connected to a 2 in long neoprene tubing and closed with a luer-lock cap at the end. The neoprene tubing was secured

with a clamp to control the liquid flow when taking the samples because of the over pressure in the inside of the reactor (Figure A-2).

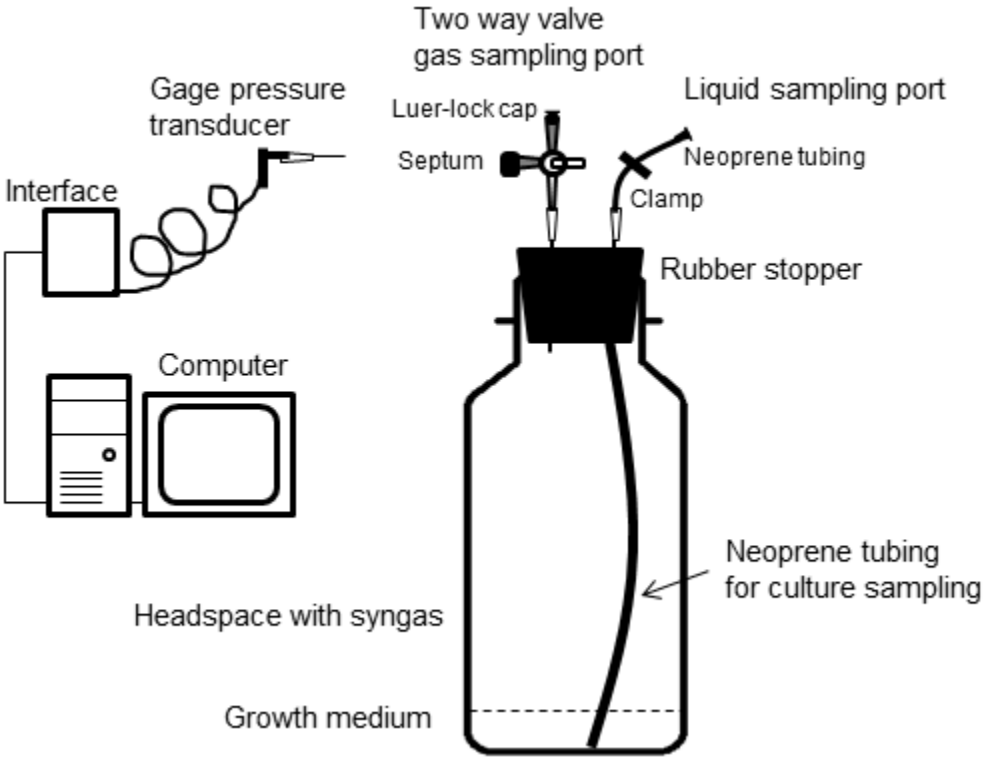



**Figure A-2. Reactor configuration for syngas fermentation experiments with limited syngas supply**

A second needle that was punched through the rubber stopper was used to measure inside gas pressure and take gas samples for measuring its composition. The outer part of the needle was connected to a 2-way valve. One inlet of the valve was used to take the gas samples and measure the gas pressure and was equipped with a rubber septum. The other inlet was closed with a luer-lock cap (Figure A-2). Gage pressure transducers (Model PX26, Omega Engineering, Inc.) which were attached to hypodermic needles, were used to measure the pressure inside the reactor. The pressure transducers were connected to a computer through an interface, and the pressure data

converted to volume gas at standard temperature and pressure (STP), according to the ideal law of gases.

The reactors were placed on a shaker into an incubator and the temperature was set at 35ºC. At the moment of the gas pressure sampling, the shaker was stopped and the gage pressure transducers connected to the two way valve by punching the needle through the septum. The shaker was stopped to prevent gas leakage through the septum when the needle was connected. To equilibrate the temperature after opening the incubator's door, half an hour after the connection of the pressure transducers the pressure was recorded. 500 micro liter of gas was sampled using a gastight Hamilton sample lock glass syringe and analyzed using gas chromatography. The pressure in the syringe was equilibrated with the ambient pressure by placing the extreme of the needle in a beaker with water and unlocking the syringe, then locking it again to keep the sample inside. The sample was injected in the GC immediately to avoid leakage.

***Culture of* Clostridium ljungdahlii *in serum bottles for inoculum source purpose***

*Medium preparation (ATCC 1756)*

Stock solutions for minerals, vitamins, and trace metals were prepared separately

**Mineral stock solution**

For 1 liter solution, pour 900 ml DI water in a beaker and add the following chemicals:

- sodium chloride 80 g
- ammonium chloride 100 g
- potassium chloride 10 g
- potassium phosphate monobasic 10 g
- magnesium sulfate 20 g
- calcium chloride 4 g

Adjust the final volume to 1 L with DI water

**Vitamin stock solution**

For 1 liter solution, pour 900 ml DI water in a beaker and add the following chemicals:

- pyridoxine 0.01 g
- thiamine 0.005 g
- riboflavin 0.005 g
- calcium pantothenate 0.005 g
- thioctic acid 0.005 g
- amino benzoic acid 0.005 g
- nicotinic acid 0.005 g
- vitamin B12 0.005 g
- biotin 0.002 g
- folic acid 0.002 g

- MESNA 0.01 g

Adjust the volume to 1 L with DI water

**Trace metals stock solution**

For 1 liter solution, pour 900 ml DI water in a beaker and add the following chemicals:

- nitrilo triacetic acid 2 g
- manganese sulfate 1 g
- ferrous ammonium sulfate 0.8 g
- cobalt chloride 0.2 g
- zinc sulfate 0.2 g
- copper chloride 0.02 g
- nickel chloride 0.02 g
- sodium molybdate 0.02 g
- sodium selenite 0.02 g
- sodium tungstate 0.02 g

Adjust the volume to 1 L with DI water

**Medium preparation**

For a 250-ml total capacity serum bottle (20 ml of medium and 230 ml headspace), pour 15 ml DI water in a beaker and add the following solutions and chemicals:

- Mineral solution 0.6 ml (30 ml/L)

- Trace metals solution 0.2 ml (10 ml/L)
- Yeast extract 0.01 g (0.5 g/L)
- MES sodium salt 0.65 g (32.58 g/L or 150 mM)

Adjust pH to 5.5 by adding KOH 5 M solution

Add DI water to complete 19.6 ml

Pour the solution in the serum bottle (250 ml) and flush the medium with nitrogen for 20 min using a long (6 in) needle and a rubber stopper in the opening to prevent air to come in. Take the needle out and seal the bottle with a crimp seal. Connect the bottle to a syngas outlet using a needle and flush the headspace for 5 min using another needle as an exhaust. After 5 min take the exhaust needle out and let the pressure equilibrate with the pressure in the line (14 psi). Autoclave the bottle (Set the time according to the autoclave instructions)

Let the bottle and medium cool down and add the following solutions (always work close to a flame or in a biological hood. Clean the stopper with ethanol before inserting a needle):

- Vitamin solution 0.2 ml (10 ml/L)
- Cysteine sulfide 2.5% 2.2 ml (10 ml/L)

Let the cysteine sulfide work for 5 min before inoculation.

Inoculate 10% v/v with a living culture.

**Medium preparation for precultures without yeast extract**

Experiments where no yeast extract is used require a preculture grown without yeast extract in growth medium to prevent any remaining yeast extract to contaminate the experiment. For this

purpose, prepare the growth medium following the same instructions explained above, except the addition of yeast extract in the solution.

***Protocol for running GC for evaluation of individual Solvents***

*Sample Preservation and Storage:*

• Samples should be filtered (0.22 μm) to remove any suspended solids

• Samples should be diluted to <~1.0 g/L solvent if possible

• Measure samples immediately, if possible. Filtered samples can be stored for up to 1 week in a sterile container at 4ºC if necessary.

*Standards*

Standard stock solution should be 50 mM of acetone, ethanol, propanol, *n*-butanol, pentanol, hexanol, 2-methyl-1-propanol, and standards should be made at 1, 7, 21, 30, and 50 mM, respectively.

*Start-up*

1. Turn on the air, helium, and hydrogen cylinders, and turn the flow on at the GC (left hand side panel).

2. If you suspect the flow rate has changed, check it with the bubble meter.

3. Turn the switch on the right side (when facing the front) of the machine on.

4. Turn on the communication module (separate unit to the left of the GC labeled as alcohol GC).

5. On the Windows 98 computer, open “Instrument 1 (online)” from the desktop.

6. Under the menu “Sequences” open “Sequence Table”

7. Click “Insert Vial Range” and put in the range of vials you will run on the autosampler (Method is: SOLVENTS, Injections: 1, Injection volume: 1). Click “OK”.

8. Under the menu “Sequences” open “Sequence Parameters”. Put in the name of the directory in which you want to store files. Click “OK”.

9. Under the menu “Sequences” click on “Save Sequence” and give it a name (you can reuse the same file later by opening it and changing the sequence table and re-saving.

10. Wait until GC reaches operating temperature (15-25 min).

11. Push the Ignite button (on left panel of GC) to light the flame (if you don’t hear it light, blow slightly in the exhaust port and it will light).

12. Allow 15 min to equilibrate.

13. Check that the washing vials are fresh with clean water and empty the waste vials

14. If computer screen reads “Ready” then click “Start”.

*Injections*

1. Check the syringe to make sure it is functioning properly

2. Injections should be with 2 mL and quick

*Shutting Down*

1. Shut off the program on the computer, and turn off the communication module.

2. Turn off the hydrogen valve at the machine to extinguish the flame.

3. Push [Oven Temp] and set to 25, set [DetA Temp] to 50, and [InjA Temp] to 50.

4. After components have cooled, turn off the GC, then turn off the air and carrier valves.

5. Turn off the gas cylinders

*Data Analysis*

1. Open "Instrument 1 (offline)" on the computer

2. Go to "File" and "Load Signal", and load your first standard vial

3. Go to "Calibration" and click "Add Peaks" and put in the calibration level (concentration) when prompted.

4. Remove (delete) unwanted peaks from the calibration table.

5. Put in the names of the peaks

6. Load a new standard vial and under "Calibration" click on "Add Level". The program should automatically recognize the proper peaks.

7. Once you have filled your calibration table, you can load your data signals, one at a time.

8. Click on the magnifying glass at the top right, which should bring up the report for your vial, with concentrations of the VFAs.

9. Until further notice, you have to write these down, we haven't figured out yet how to export the data to excel.

***Protocol for running GC for evaluation of individual VFAs***

*Sample Preservation and Storage:*

• Samples should be filtered (0.22 um) to remove any suspended solids

• Dilute sample to ~500 mg/L total VFA (as acetate) with 2% formic acid

• For storage, filtered samples should be diluted at least 1:1 with 2% formic acid to lower the pH, and stored in 4oC refrigerator for subsequent analysis

*Standards*

The stock solution of volatile fatty acids contains 10mM each of: formic, acetic, propionic, isobutyric, *n*-butyric, isovaleric, *n*-valeric, isocaproic, *n*-caproic, and *n*-heptanoic acids. Dilute the stock solution to make injection standards of .5, 1, 2, 5, and 10 mM. Standards should be run weekly at least.

*Start-up*

1. Turn on the air, helium, and hydrogen cylinders, and turn the flow on at the GC (left hand side panel).

2. If you suspect the flow rate has changed, check it with the bubble meter. The flow of air, carrier (He), makeup (He), and hydrogen should be: , respectively.

3. Turn the switch on the right side (when facing the front) of the machine on.

4. Turn on the communication module (separate unit to the left of the GC).

5. On the Windows 98 computer, open "Instrument 1 (online)" from the desktop.

6. Under the menu "Sequences" open "Sequence Table"

7. Click "Insert Vial Range" and put in the range of vials you will run on the autosampler (Method is: VFATRIAL, Injections: 1, Injection volume: 1). Click "OK".

8. Under the menu "Sequences" open "Sequence Parameters". Put in the name of the directory in which you want to store files. Click "OK".

9. Under the menu "Sequences" click on "Save Sequence" and give it a name (you can reuse the same file later by opening it and changing the sequence table and re-saving.

10. Wait until GC reaches operating temperature (15-25 minutes).

11. Push the Ignite button (on left panel of GC) to light the flame (if you don't hear it light, blow slightly in the exhaust port and it will light).

12. Allow 15 minutes to equilibrate.

13. Check that the washing vials are fresh with clean water and empty the waste vials

14. If computer screen reads "Ready" then click "Start".

*Injections*

1. If the autosampler gets stuck, it is usually a syringe problem. Remove the syringe, make sure the plunger moves freely, and replace. Restart the sequence.

*Shutting Down*

1. Shut off the program on the computer, and turn off the communication module.

2. Turn off the hydrogen valve at the machine to extinguish the flame.

3. Push [Oven Temp] and set to 25, set [DetA Temp] to 50, and [InjA Temp] to 50.

4. After components have cooled, turn off the GC, then turn off the air and carrier valves.

5. Turn off the gas cylinders

*Data Analysis*

1. Open "Instrument 1 (offline)" on the computer

2. Go to "File" and "Load Signal", and load your first standard vial

3. Go to "Calibration" and click "Add Peaks" and put in the calibration level (concentration) when prompted.

4. Remove (delete) unwanted peaks from the calibration table.

5. Put in the names of the peaks

6. Load a new standard vial and under "Calibration" click on "Add Level". The program should automatically recognize the proper peaks.

7. Once you have filled your calibration table, you can load your data signals, one at a time.

8. Click on the magnifying glass at the top right, which should bring up the report for your vial, with concentrations of the VFAs.

9. Until further notice, you have to write these down, we haven't figured out yet how to export the data to excel.

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